Screening of endophytic diazotrophic bacteria in wheat under saline conditions from central regions of Uttar Pradesh

Thesis

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BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
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CERTIFICATE

This is to certify that the thesis titled **"Screening of endophytic diazotrophic bacteria in wheat under saline conditions from central regions of Uttar Pradesh"** submitted by **Ms. Sushma Verma** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (Ph.D.) regulations - 1999 as amended in 2008/2010/2013 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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DECLARATION

This is to certify that I have worked on the research thesis entitled **"Screening of endophytic diazotrophic bacteria in wheat under saline conditions from central regions of Uttar Pradesh"**. The data mentioned in this thesis were collected and obtained during genuine work done by me. Data obtained from other agencies have been duly acknowledged. None of the findings pertaining to the work has been concealed. The result embodied in this report has not been submitted to any other University, Institution or Research Centre for the award of any degree.

Place: LUCKNOW

Date: 30/06/2022

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(Sushma Verma)

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Sushma

(Sushma Verma)

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% Percent

$(NH_4)_2SO_4$ Ammonium Sulphate

°C Degree Celsius

°E Degree East

°N Degree North

µg Micro Gram

µL Micro Liters

µS/cm Micro-Siemens per Centimeter

µS/m Micro-Siemens per meter

ACC 1-Aminocyclopropane-1-Carboxylate

ACCD 1-Aminocyclopropane-1-Carboxylate Deaminase

ANOVA Analysis of Variance

BLAST Basic Local Alignment Sequence Tool

BNF Biological Nitrogen Fixation

bp Base Pairs

BTB Bromothymol Blue

CAS Chrome Azurol S

CFU Colony Forming Unit

cm Centimeters

CMC Carboxymethylcellulose

DAS Days After Sowing

DF Dworkin and Foster

DMRT Duncan’s Multiplicity Range Test

DNA Deoxy Ribonucleic Acid

dS/m Deci-Siemens per meter

EC Electrical Conductivity

g Gram

GPA Glucose Peptone Agar

H_2O_2 Hydrogen Peroxide

H_2S Hydrogen Sulphide

ha Hectare

HCN Hydrogen Cyanide

HDTMA Hexadecyltrimethylammonium

hrs Hours

IAA Indole-3-Acetic Acid

K Potassium

kDa Kilo Dalton

Kg Kilogram

KNO3 Potassium Nitrate

m Meter

mg Miligram

Mha Million Hectares

ml Mili Liters

MR-VP Methyl-Red and Voges-Proskauer

N Nitrogen

N2 Dinitrogen

Na+ Sodium Ion

NaCl Sodium Chloride

NaNO3 Sodium Nitrate

NaOH Sodium Hydroxide

NBAIM National Bureau of Agriculturally Important Microorganisms

NH3 Ammonia

NH4Cl Ammonium chloride

nm Nanometer

OD Optical Density

PDA Potato Dextrose Agar

PGP Plant Growth Promoting

PGPB Plant Growth Promoting Bacteria

PGPR Plant Growth Promoting Rhizobacteria

PGPE Plant Growth Promoting Endophytes

PSB Phosphate Solubilizing Bacteria

PSI Phosphate Solubilization Index

PSU Percent Siderophore Unit

rpm Rotation per Minutes

rRNA Ribosomal Ribonucleic Acid

SD Standard Deviation

SEM Scanning Electron Microscopy

ST-PGPR Salt-Tolerant Plant Growth Promoting Rhizobacteria

UV Ultra Violet

UV-Vis Ultra Violet Visible

w/v Weight by Volume

WHO World Health Organization

YEMA Yeast Extract Mannitol Agar

Zn Zinc

ZSI Zinc Solubilization Index

α Alpha

β Beta

θ Theta

ϒ Gamma

INTRODUCTION

Environmental degradation is an alarming issue and has emerged as a major concern to humanity over the past few decades. Mindless consumerism, economic growth along with perpetual human interferences are continuously worsening the scenario of planet's habitability systems and exhibit detrimental effects on Mother Nature; though in spite of this, the pace and desire for economic development have never ceased. High levels of poverty and chronic malnutrition confronts the limitations in human capital development and its high time to remind ourselves that economic growth is only sustainable if all countries have food security strategies. Food security is a major tool to end hunger along with improving nutritional status which not only carries significant benefits for human health but has also been persisted as a key point to reach sustainable economic development accompanied by on-going land system changes on the planet (Bonis-Profumo et al., 2019). Relatively, solving food security is catalogued as one of the prime challenges under the second pillar of "zero hunger" of 17 sustainable development goals set up by the United Nations (Hambrey, 2017; Subramaniam and Masron, 2021; Fig 1).

Fig.1 United Nations Sustainability Development Goals (SDGs) (Izzo et al., 2020)

World's agricultural system is manifested by plethora of challenges in twenty-first century such as deprivation in agroecosystem sustainability and lessened productivity. There has been a continuous upsurge in the demand for food, and a concurrent inadequacy in its supply as the global human population is expected to reach approximately 9 billion by 2050 according to the estimates of the United Nations (Prasad et al., 2019). Climate-smart agriculture plays a central role in ensuring the food security by safeguarding region's food supply as it directly affects the environment through its impacts on biogeochemical cycles as well as agroecosystems. Agricultural systems also influence the natural habitats through land conversion, eutrophication, excess pesticide inputs, irrigation, drainage and unsustainable agronomic practices. Soil degradation is defined as the transformation of soil quality status that encompasses erosion, salinization, and loss of soil due to deforestation, overgrazing, soil compaction/crusting, and waterlogging (Ayub et al., 2020). The application of chemical fertilizers in excess could also lead to serious loss of soil carbon aggravating the decline of soil organic matter and fertility. Various abiotic constraints such as salinity, drought, extreme temperature reduces productivity on both irrigated and non-irrigated agricultural lands by causing severe damage to overall soil health throughout the world (Gregorio et al., 2013; Mishra et al., 2017a; Ma et al., 2020). In recent years soil salinization is becoming one of the major concern for researchers and governments across the globe as it imposes osmotic stress, ion toxicity, nutrient (N, Ca, K, P, Fe, Zn) deficiency, oxidative stresses on plants, limits water uptake from soil and ultimately negatively impact the productivity of major crops (Arora et al. 2012; Nadeem et al. 2016).

Salinity is an active process escalating globally and is considered as one of the most significant causes of land degradation in arid and semiarid regions; however, the pace to mitigate salinity in developing world is much slower (Kumar et al. 2015). At present about 1 billion hectares of land worldwide is suffering from the problem of salinity/sodicity stress of which 351.2 million hectares (Mha) comes under salt stress and 581.0 Mha are sodic (Arora et al., 2018; FAO 2020). Relatively, Orhan (2016) also reported that 15% of the total saline soils falls in arid and semi-arid areas while 40% are found to be on irrigated lands. With this ever-increasing trend of the level of salt affected areas it is timely important to identify and discuss what the developing countries can learn from the sustainable approaches of developed world and opt for suitable alternatives in order to tackle soil salinity. Dry climates with low precipitations, high evaporation rate, poor drainage or waterlogging, salt transport, and ion exchange are the major contributors of soil salinization. Additionally increase in the concentrations of atmospheric greenhouse gases, consequent rise in air temperature, decline in relative humidity together with the extreme rainfall events are some of the probable indicators of climate change and do also have a huge impact on the pace of soil salinity development (Haj-Amor et al., 2020). In the same regard maintenance of dynamic nature of soil has become one of the urgent needs which will assuredly not only to mitigate the demands of ever-increasing population but will also sustain our agricultural ecosystem from environmental distresses (Paustian et al., 2016).

Microorganisms serve as an important part of the soil ecosystem and are characterized by high genetic diversity. Rhizosphere is the zone of soil that surrounds the plant root and serves as the hot spot of unique population of microorganisms. Soil microbes play foremost role in the ecology of soil and are widely involved in soil organic matter decomposition, production of plant growth regulators, synthesis of volatile organic compounds (VOC), promotion of shoot and root growth, disease control or suppression, production of secondary metabolites, and improving soil structure. One of another significant metabolic activities of them is nutrient management, and recycling of the elements onto which the main productivity depends on. Amelioration of salinity stress through the application of valuable soil microbes is also considered as a promising approach of a sustainable agriculture because of less side effects and eco-friendly nature.

Nitrogen (N) serves as a major component of amino acids (the building blocks of proteins) and it is also a vital component of chlorophyll utilized by plants for photosynthesis (Uchida 2000, Nunes-Nesi et al., 2010; Egamberdieva et al., 2020). N is essential to life: a key building block of DNA and plays a very important role in plant growth, development, and reproduction. N eventually used by plants comes primarily from two natural sources: a vast storehouse of nitrogen in the atmosphere and the nitrogen-containing minerals (Tipping et al., 2017). Despite the fact that it is one of the elements found on earth in ample amount, N deficiency is undoubtedly the most common nutritional problem of plants (Roy et al., 2006). The element in the soil basically exists in three general forms: ammonium (NH_4^+) ions, nitrate (NO_3^-) ions, and organic N compounds. The majority of the nutrient that is available to plants lies in the inorganic forms NH_4^+ and NO_3^- (mineral nitrogen), whereas the soil N from the atmosphere and earth's crust that is in organic form is not directly accessible to primary producers such as plants (Bowman 2020). A very small amount of organic nitrogen exists in soluble state in the form of organic compounds, such as urea which may be slightly available to plants (Lewis et al., 2011; Kumar et al., 2019). Nutrients go through a lot of transformations in the soil and all these alterations are often clustered into a system known as the nitrogen cycle. It is well reported that most of these transformations are due to the activity of microorganisms such as bacteria, archaea, and fungi and involve several processes such as nitrogen fixation, nitrification, immobilization, and denitrification (Robertson and Groffman, 2007; Egamberdieva, 2011). Only a selected group of prokaryotic microbes are able to carry out these energetically demanding processes (Bernhard, 2010). Although mostly the plants are dependent for N fixation on prokaryotes, some N can also be fixed abiotically by the effects of lightning, and in industries primarily by the Haber-Bosch process, that requires a high level of fossil fuels combustion. The total contribution of the fixed nitrogen to the earth's surface has been projected to be about 100 million tons per year, out of which 90 percent of N is considered to be fixed biologically (Donald 1960; Burns and Hardy, 1975). The process of nitrogen fixation occurs both biologically and non-biologically and is defined as "the phenomenon by which molecular nitrogen in the air is transformed into ammonia (NH_3) and related nitrogenous compounds in soil that is readily available for assimilation by plant and microbes" (Gresshoff et al., 2015). When the fixation occurs with the aid of soil microorganisms it is called as biological nitrogen fixation (BNF) which was early discovered by Dutch microbiologist Martinus Beijerinck (Fatima et al., 2019). It involves the representatives of various bacterial phylogenetic groups, which are collectively called as diazotrophs (Figueiredo et al., 2013). The term 'diazotroph' originates from diazo and troph which signifies "two nitrogen" (or dinitrogen) and "pertaining to food", respectively and is defined as microorganisms especially bacteria that fixes the atmospheric nitrogen into a more usable form such as ammonia (Deveryshetty and Antony, 2021, Postgate, 1998). BNF can either be symbiotic (also called associative, or nodulating fixation) that happens when there are mutualistic associations between plant species and nitrogen fixing microorganisms (predominantly rhizobia). The asymbiotic BNF (also called as free-living or non) offers great potential for accelerating crop production and is accomplished by free-living nitrogen fixing microorganisms, such as species of the genera *Beijerinckia* and *Azotobacter* (de Freitas 2022; Reed et al., 2011; Granhall, 1981). It is projected that global BNF adds 122 Tg of N yearly of which cultivated agricultural systems fix approximately 33 to 43 Tg (Figueiredo et al., 2013; Adams et al., 2018). In agricultural ecosystems root nodule-bearing legumes undoubtedly are the prominent nitrogen fixers followed by blue-green algae and other plant growth promoting microorganisms (PGPM). These N fixing microorganisms stimulate the plant growth not only by providing N but also by other mechanisms like siderophore production, secretion of exopolysaccharides (EPS), and phytohormones; phosphate solubilization; and protection against deleterious fungal phytopathogens (Dakora 2003; Moreira et al. 2011). Microbes possess an enzyme complex known as nitrogenase that is responsible for BNF and the enzyme system is composed of two major protein components named as, dinitrogenase (MoFe-protein) and dinitrogenase reductase (Fe-protein). Both the components are associated with metalloclusters and are regulated by a set of multiple genes (Dighe et al., 2010). The structural subunits of homodimeric Fe-protein are encoded by the *nifH* gene, and the heterotetrameric MoFe-protein is encoded by the structural genes *nifD* and *nifK*. The nitrogenase enzyme system catalyzes the reduction of N_2 to NH_3 accompanied by reduction of protons to H_2 and the process is coupled to the hydrolysis of 16 equivalents of ATP. The BNF can be depicted by the formula:

$$N2 + 6\ H+ + 6\ e- \rightarrow 2\ NH3$$

The *nifHDK* genes of conventional nitrogenase are often found in contiguous arrangement within the genome (Zehr and Turner, 2001). The genes can be detected and characterized by amplifying the environmental samples using the polymerase chain reaction (PCR).

Multifaceted interaction between plants and endophytes involves a well-versed functional relationship and apart from commensalistic symbionts, microorganisms may exist from latent pathogenic or saprotrophs to mutualistic associations. For example, in rhizobia-legume symbiosis, rhizobial cells are transformed into bacteroids that fix atmospheric N into ammonia. The symbiosis is signified as renewable source of N contributing a big share in total N economy of the globe (Krapp et al. 2011; Rajwar et al. 2013). In addition to rhizobia other endophytes are also able to fix N and promote plant growth, elicit defense response against pathogen attack, and can be helpful in the remediation of abiotic stresses. The term “endophyte” was first introduced by De Bary (1866) and he defined them as “any organism that grows within plant tissues are termed as endophytes,” However, the definition continued to evolve (Wilson, 1995; Bacon and White, 2000). Endophytes encompass all the culturable and unculturable microorganisms that inhabit the interior of plant tissues at some part of its life cycle without causing any harm to the host plant (Petrini 1991; Khare et al., 2018). Microbial endophytes are present in all known species of plants and the relationship that they establish ranges from symbiotic to bordering on pathogenic (Khare et al., 2018). Microbes dependent on the plant metabolism for survival and being spread amongst plants by the activity of different types of vectors are termed obligate endophytes (Hardoim et al., 2008). However, the facultative endophytes are mostly associated with plants from its neighboring soil environment and live outside the host plant for certain stages of their life cycle (Abreu-Tarazi et al., 2010; Gouda et al., 2016). Endophytes play important role in promoting the plant growth through the production of plant growth enhancing substances such as indole acetic acid (IAA), cytokinins, gibberellins, siderophores, phosphorus solubilization, secretion of phytoharmones, atmospheric N fixation, improving nutrient acquisition, and supply of essential vitamins and enzymes (Xia et al., 2015; Vardharajula et al., 2017). Compared with other rhizospheric microbial communities, endophytes are more likely to interact more closely with their host plant tissues escaping competition with rhizosphere microorganisms. Some of the possible mechanisms exhibited by them to stimulate plant growth under environmental stresses comprises of synthesis of phytohormones (abscisic acid, gibberellic acid, cytokinin's, and indole -3-acetic acid (IAA), production of ACC deaminase, induced systemic tolerance, accmulation of osmolytes, and involvement of antioxidant defense systems (Fig. 2).

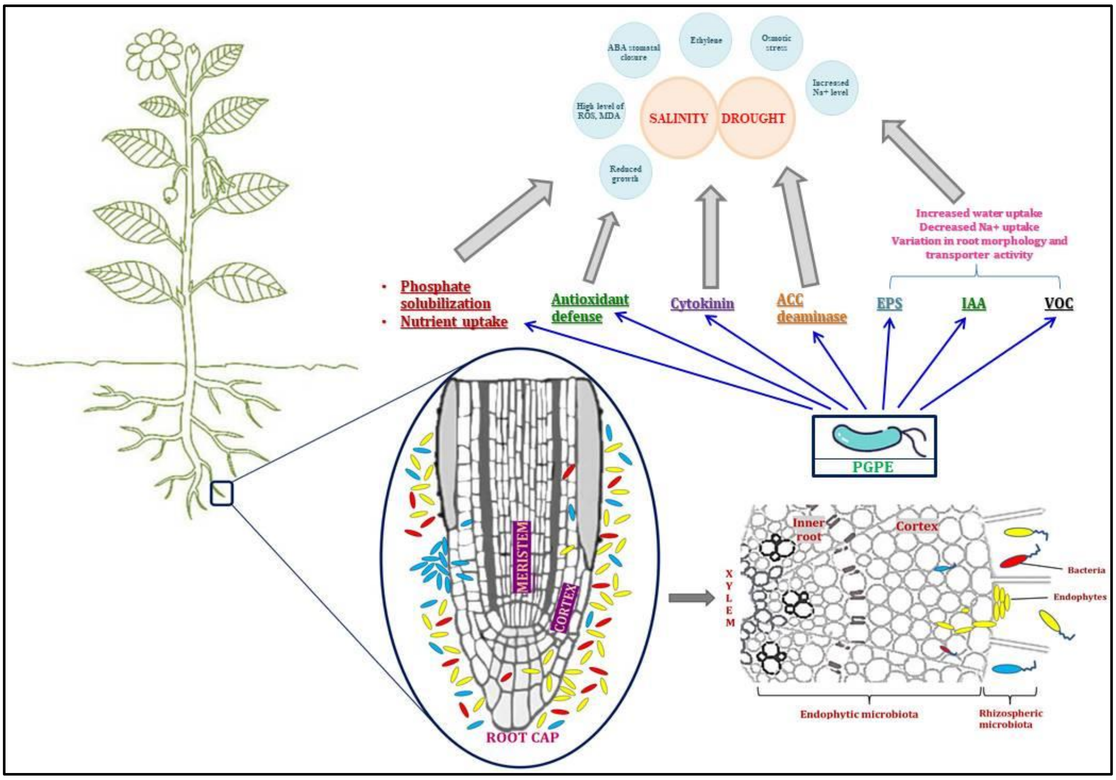


Fig.2 Plant growth promoting endophytes (PGPE) and salinity

Cereals are grasses from the Poaceae family and amongst them, maize, rice, wheat, and sorghum are considered as the most important nutritious crops consumed by humans. The cereal crops and their products are considered to be the most important source of many nutrients for both children as well as adults (Laskowski et al., 2019). Wheat (*Triticum aestivum* L.) is one of the major staple cereal crop, renewable resource for food, feed, and industrial raw material, protein, and fiber source in the human diet, and is grown both as spring and winter crop (Ramadoss et al., 2019; Babbar et al., 2022). Most of the cereal crops including wheat are reported to be deficient in micronutrients which not only reduces the plant yield but also lessen the absorption of certain significant nutrients by humans leading to various diseases and ultimately endangered public healthcare systems (Akbarabadi et al., 2015; Dixit and Yadav, 2020). The history of wheat cultivation is closely related to the past of the changing relationship of humans to their environment, and especially the efforts to protect themselves from hunger and get control over its food supply (Igrejas and Branlard, 2020). The crop ensures the food security of almost 57 percent of the world's population; however recent concerns have highlighted the critical role of the cereal in nutritional requirements of the poor and vulnerable. Wheat is known to be a widely adaptable crop and can be propagated in assorted climates of India ranging from tropical to temperate as well as cold northern parts. The crop is cultivated mainly across six diverse agro-climatic zones i.e., Northern hills zone (NHZ), North western plains zone (NWPZ), North eastern plains zone (NEPZ), Central zone (CZ), Peninsular zone (PZ) and Southern hills zone (SHZ). The zones have been classified on the basis of soil types, climatic conditions, and growing durations of wheat cultivars. Time of sowing varies from zone to zone and timely sowing paralleled with following the sustainable agronomic practices may help in achieving the high yields of wheat. Statistical data shows that the production volume of wheat amounted to over 765 million metric tons globally in the marketing year of 2019-2020 and main producers involved in production of wheat worldwide has been depicted in Fig. 3 (https://www.statista.com/statistics/237912/global-top-wheat-producing-countries/).

The crop has been reported to be under cultivation in about 30 million hectares in India with an approximately recorded average productivity of 3371 kg/ha in India (MoA&FW, 2018).

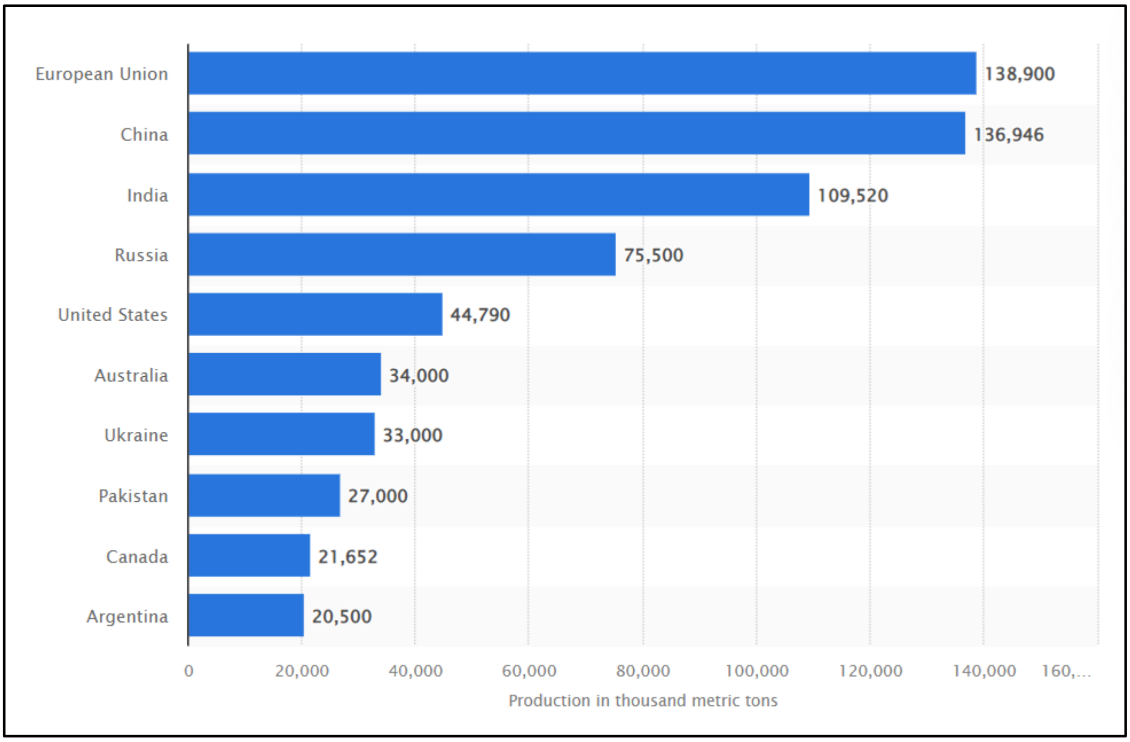


Fig.3 Ten leading producers in the world

(Source: https://www.statista.com/statistics/237912/global-top-wheat-producing-countries/)

Abiotic stresses are considered to be responsible for approximately a 50% decrease in crop productivity, imposing a serious threat food security at global level (Acquaah, 2007; Seleiman, 2019). However, increasing levels of such environmental stresses especially salinity on irrigated lands is a challenge for wheat production adversely affecting seed germination, plant growth, photosynthesis, water relations, nutrient uptake, and yield of wheat (Fig. 4). Salinity, polygenic character regulated by multiple genes affects 20% of cultivable land globally and is increasing constantly owing to the change in climate and anthropogenic activities (Arora, 2019). Literature states that about 4.5 million hectares of salt-affected area come under wheat agronomy that will significantly reduce the yield of the crop (Singh et al., 2010). Most of the wheat that is cultivated is hexaploid that has some acquired tolerance to salinity stress. Plants do have stress-specific adaptive responses and some of the strategies primarily utilized to prevent as well as adapt to high Na+ concentrations involve: i) activation of Na+ efflux, (ii) Na+ influx inhibition and (iii) Na+ compartmentalization in vacuoles. The options to raise productivity are very limited when the plants have to be cropped on saline and sodic soils. Various techniques such as screening and selection of suitable genotypes, introduction of desirable genes along with conventional breeding techniques have been used globally to improve the performance of crops growing under high salt environments; however, these approaches are costly as well as time-consuming (Hassan et al., 2018; Adrees et al., 2020). Therefore, coordinated attempts are essentially required by the researchers to study adaptive mechanisms such as use of osmoprotectants, seed priming, nutrient management, along with hormonal application and develop eco-friendly strategies to manage salinity stress in wheat.

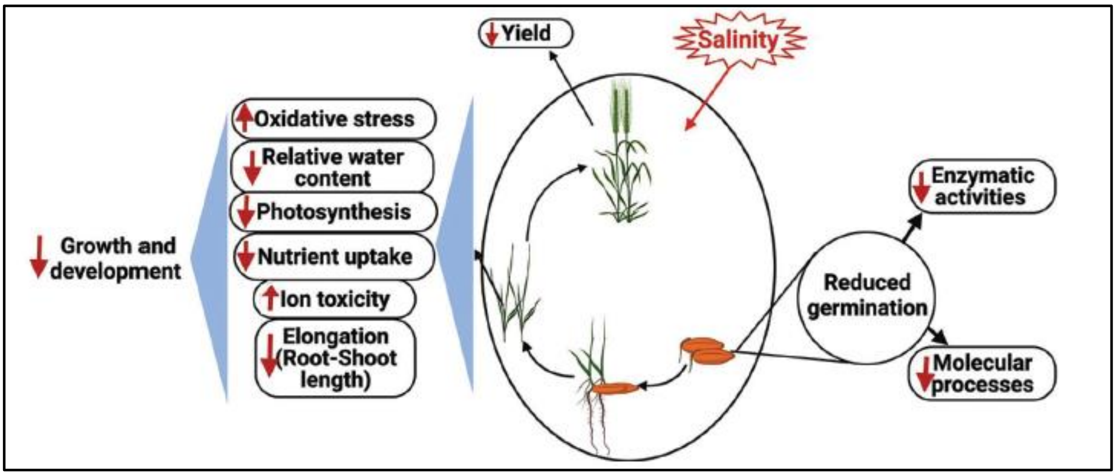


Fig. 4 Effect of salinity stress on wheat crop (Seleiman et al., 2022)

Endophytes and their association with various plants growing under stressed conditions is an important aspect of research and to figure out them in detail it is highly needed to explore their phenotypic and genotypic diversity. The present study is mainly focused on the multidimensional connections between root endophytes associated with wheat, particularly, in relation to maintaining the health of the plant under normal as well as saline conditions. The work roughly involves isolation, screening, characterization, and further application of selected endophytic isolates to its respective host plant i.e., wheat. This will not only help in elucidating the diversity of N fixing endophytic bacterial microflora but will also help in understanding their approach to survive and promote growth of the plant in the environment exposed to salt stress.

Objectives:

1. Collection of wheat plants from salt affected soils.
2. Isolation of endophytes from procured plants.
3. Identification of endophytes and their characterization.
4. Screening of diazotrophs amongst isolated endophytes.
5. *In vitro* studies to assess the impact of diazotrophic endophytes on the growth of host plant.

REVIEW OF LITERATURE

Climate change is a significant threat to the productivity of the world's agricultural systems which is becoming a rapidly growing concern worldwide (Arora, 2019). Increased global temperatures will elevate the levels of ocean as polar ice caps melt and will bring more extreme weather events around the globe (Hansen et al., 2016; Corwin 2021). The framework that is being followed at present for "business-as-usual" farming predicts that the food security challenges will exceedingly increase by 2050 (Smith et al., 2013). It is also anticipated by many researchers that the growth in population and income leading to intensification in demand for food and other agricultural commodities will enormously upsurge the production of coarse grains, rice, wheat, oilseeds, and sugar by around 70 percent in coming decades (Wiebe et al., 2019). Malnutrition causes large economical losses and widespread human sufferings (Pinstrup-Andersen, 2013). Keeping all this in mind considerable efforts are required to increase the plant tolerance to various abiotic and biotic stresses to improve the gains in productivity and quality. This will not only ensure the access of sufficient quantity of affordable, nutritious food to the fast-growing world population but expectantly will also to be able to meet the predicted demands for cereal crops in the near future.

2.1 Nitrogen

Green plants have an exceptional capability of reducing carbon in photosynthesis and thereby they are believed to be unique among all the living organisms. Although all the forms of life require carbon as energy source, N is also one of the most significant nutrients because of being a vital component of proteins enzymes, chlorophyll and plant growth regulators, etc. (Novoa et al. 1981). Proteins act as catalyst in various important enzymatic processes as well as they also act as storage mediums to fulfil the nutritional demands of seedlings of developing plants (Ougham et al. 2008; Rasheed et al., 2020). Nucleic acids are the most important of all naturally occurring biomolecules that code, store, and translate the genetic information the cell needs to make proteins in biological systems (Minchin and Lodge, 2019). One of the challenging topic to the researchers is plant N nutrition that deals with a number to complications not encountered in other areas of plant nutrition research (Näsholm et al., 2009). Soil organic N serves both as a significant mineralization substrate and as a direct source of N for variety of plants in various ecosystems (Bennett and Prescott, 2004; Hawkins et al., 2005). It is an indispensable element for all organisms, and a constituent of proteins, nucleic acids (DNA, RNA) and other essential organic compounds (Soetan et al., 2010; Ohyama 2010). Nitrogen signifies about 2 % of the total plant dry matter which enters the food chain. Availability of N is considered to be a major factor to the plants which determines their growth and productivity at later stages. The quantity of N needed for agriculture is projected to increase in the next decades as the plants are unable to get this nutrient directly from air as every nitrogen atom is triple-bonded to another nitrogen atom to form molecular nitrogen, N_2 (Gupta et al., 2012). Even though the presence of dinitrogen gas in the atmosphere in abundance, the element is commonly deficient in agricultural soils in its oxidized and reduced forms and is available to plants generally in the form of ammonium and nitrate. The fixed form is generated by the conversion of N2 into a combined form i.e., NH3 and the process is called as nitrogen fixation that occurs by means of chemical and especially biological action.

2.2 Biological Nitrogen fixation

The intensive use of nitrogenous chemical fertilizers during agricultural practices has caused serious environmental degradations leading to an unprecedented perturbation of the nitrogen cycle (Olivares et al., 2013; Rahman et al., 2021). Besides this increased use efficiency it is anticipated that priority should be given to put on value to the process of BNF of the agricultural crops that will reduce the undesired effects of chemical N fertilization. The biogeochemical cycles of all the elements are interrelated so that the alteration of the N cycle cannot be considered separately from other elements such as carbon (Gruber and Galloway 2008). Nitrogen fixation is an essential process that greatly affects the primary productivity of plants as the fixed inorganic nitrogen compounds are importantly needed for the biosynthesis of all nitrogen-containing organic compounds (Fig. 5). Approximately 425 Tg of reactive nitrogen is fixed globally every year as a result of several natural and human activities (Sutton and Bleeker, 2013). The role of N in global food production is indisputable and it is also evident that ever-increasing amounts of synthetic N are being applied annually in the form of fertilizers to crop plants (Singh, 2018). Their application has caused frequent ecological and environmental problems, such as hardening of soil, lessened soil fertility, air and water pollution, greenhouse gas emissions, eutrophication, soil acidification, and 35 biodiversity reduction (Mcisaac et al., 2001;

Bowman et al., 2008; Arora et al., 2017). Nitrogen is fixed mainly by two vital processes i.e., biological and non-biological.

Non biological means of fixation are comprised of industrial fixation i.e., Haber-Bosch process and lightning which transforms nitrogen and oxygen into Nitrogen oxides (Bezdicek and Kennedy 1998; Roper and Gupta 2016). BNF is an essential process in the global nitrogen cycle that involves the reduction of dinitrogen from the air to ammonia with the aid of soil microorganisms. This microbiological process offers the alternative to concerns of the economic and environmental costs of the heavy use of chemical N fertilizers in agriculture (Bohlool et al., 1992; Moring et al., 2021). It accounts for roughly two-thirds of the fixed N produced on earth and is carried out by a diversified group of bacteria and archaea ordinarily termed as diazotrophs (Kneip et al. 2007). BNF is one of the crucial process next to photosynthesis and is important for the maintenance of the biosphere from both environmental as well as agricultural point of view. The phenomenon is catalyzed by an enzyme complex known as nitrogenase comprising of three subunits regulated by multiple sets of genes. Different types of nitrogenase have been reported to exist in microbes that include: the Mo-nitrogenase, the Fe-only nitrogenase, and the V-nitrogenase (Figueiredo et al., 2013; Hartmann and Barnum, 2010). The functional and structural components of the Mo-nitrogenase enzyme complex are principally encoded by ~20 genes called N-fixation genes (nif genes) and are coordinated in seven operons (nif cluster) spanning over 24 kb (Fani et al., 2000; Puri et al., 2020). These N fixing genes fall mainly into three categories i.e., structural, regulatory, and supplementary. The functional gene *nifH* encodes the Fe-protein, and is well studied for their presence/expression in comparison to other genes to explore the phylogenetic analysis of diazotrophic bacterial communities (Franche et al. 2009). Mo-Fe protein also called dinitrogenase is an alpha2beta2 tetramer encoded by *nifD* and *nifK* genes respectively (Seefeldt et al., 2009). The nif cluster of the free-living bacterium Klebsiella pneumoniae is the most studied one for nif genes that serve as a model in order to understand the regulation, synthesis, and assembly of nitrogenase enzyme (Dixon et al., 1980).

The ability to fix N is limited to certain bacteria and archaea however diazotrophs are quite widely distributed ecologically as they can sustain life in the rhizosphere and phyllosphere, inside the plant tissues, and freely in soil and water revealing considerable diversity among phylogenetic groups (Galloway et al., 2008; Young 1998). Broadly N fixing microorganisms can be classified into a) symbiotic bacteria (e.g., rhizobia and Frankia) (Zahran, 2001) and b) free living, associative and endophytic non-symbiotic nitrogen fixing bacteria such as Cyanobacteria (*Nostoc, Anabaena*), *Gluconacetobacter, Azotobacter, Azocarus, Beijerinckia, Azospirillum, Bacillus, Enterobacter, Erwinia* (Cavalcante and Döbereiner, 1988; Baldani et al., 1986; Bhattacharyya and Jha 2012). The figures of the contribution of BNF into agricultural systems have been projected at 40 TgNy-1 and 50–70TgNy-1 while the values for natural terrestrial ecosystems range from 107 TgNy-1 to 195 TgNy-1 (Marschner et al., 2011; Cooper and Scherer, 2012). Greater use of BNF is going to reduce dependency on synthetic N fertilizers that will also help in enhancing environmental quality and sustainable food production by improving the soil tilth and fertility (Fig. 6).

2.3 Plant growth promoting microorganisms and salinity

The microscopic life is the most fundamental and living component of the soil system that becomes an integral part of the crop production on the earth as soon as a seed comes into the soil (Meena et al., 2017). Light, water, carbon, and mineral nutrients are elementary needs of plants for their survival, optimal growth, reproduction, and development. Extreme conditions and unfavorable environment poses a complex set of stress conditions (extreme high or low temperature, salinity, and drought) exceedingly limiting the growth of a plant. Higher salt concentration negatively affects the growth and development of plants in the form of osmotic stress which is then accompanied by ion toxicity latterly (James et al., 2011; Rahnama et al., 2019; Khan et al., 2020). In response to high saline environments, plants usually keep the sensitive tissues away from the zone of high salinity and also exude ions away from the cytoplasm of physiologically active cells (Silva et al., 2010).

Microorganisms are the natural inhabitants of diverse environments possessing enormous metabolic capabilities to mitigate various abiotic stresses such as drought, salinity, low or high temperatures, and other environmental extremes (Fig. 7). Their intrinsic metabolic and genetic capabilities make them suitable organisms to combat extreme environmental conditions (Singh et al., 2014). These adverse ecological aspects are amongst the principal limiting factors responsible for declining agricultural productivity and the subsistence nature of farming (Grayson, 2013). The role and function of microorganisms in the alleviation of abiotic stresses in plants has been an area of great concern to many researchers in the past few decades (Nadeem et al., 2014; Souza et al., 2015; Tewari and Arora 2014). Bacteria capable to actively colonize the plant root systems and promote plant growth are referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1981; Prasad et al., 2019). These affect the development of plants either indirectly or directly. Direct promotion of plant growth involves facilitation of the nutrient uptake from the environment as well as synthesis of metabolic compounds. Whereas indirect promotion occurs when PGPR minimizes the deleterious effects of one or more phytopathogens biocontrol, synthesizes antiobiotics, secretes extracellular enzymes and chelates available Fe in the rhizosphere (Bhattacharyya and Jha, 2012; Basu et al., 2021). PGPR can be categorized with respect to the plant compartment they occupy into two main classes namely extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR) (Martinez-Viveros et al., 2010; Vedamurthy et al., 2021). Extracellular ePGPR live either in the rhizosphere, on the root surface (rhizoplane) or in the intercellular spaces between the cells of the root cortex, whereas the iPGPR are situated inside the root cells, generally inhabiting specialized structures known as nodules.

Tolerance to salt stress is one of the significant attributes of some microbes and such rhizospheric occupants can survive in high osmotic as well as ionic stressed environments. In the last decade, various salt-tolerant plant growth-promoting rhizobacteria (ST-PGPR) procured from extreme alkaline, saline, and sodic soils have been exploited to mitigate various biotic and abiotic stresses and further improve crop production in saline agroecosystems (Arora et al., 2012; Egamberdieva et al., 2019). Various researchers have studied the diversity of ST-PGPR isolated from the rhizosphere of wheat (Rajput et al., 2019, Upadhyay et al., 2019), peanut (Sharma et al., 2016), paddy (Zhang et al., 2018). Exposure of microbes to high salt concentrations triggers rapid fluxes of cell water along the osmotic gradient out of the cell, thereby causing dehydration to counteract the outflow of water. High ion concentrations result into ionic imbalance, maintaining an osmotic equilibrium of K+, and upregulating genes assisting in adaptive, metabolic, and amino acid transport plant processes (Kumawat et al., 2022). ST-PGPR utilizes an array of processes which directly or indirectly contribute to amelioration of salinity in plants. The mechanisms of ST-PGPR includes production of phytohormones, such as auxins, gibberellins, cytokinins (Dodd et al., 2010); synthesis of ACC deaminase (Glick et al., 2007); exopolysaccharide production (Timmusk et al., 2017), and regulation of plant defense systems (Hashem et al., 2016). One of the major aspect of salt stress-tolerance in plants mediated by PGPR involves the generation of responsive machinery to pool out the toxicity and establishment of an osmotic equilibrium state. ST-PGPR constrict the Na+ uptake by changing the composition of the cell wall and increase the expression of salt overly sensitive (SOS) genes alongwith NHX transporters in plants to avoid desiccation and flaccidity in plant's cells. Accumulation of low molecular weight osmolytes (soluble sugars, amino acids, polyols and tetrahydropyrimidines) liberated by ST-PGPR also help the plants to survive in high saline conditions. It has been realized that involvement of ST-PGPR in salt stressed soil not only assist the plant survival but also improve the productivity in wide range of cereal crops (Singh and Jha, 2016; Arora et al., 2020). Upadhyay and Singh, (2015) reported ST-PGPR *B. subtilis* to enhance wheat yield by around 18% in salt-affected soil of EC 5.2 dSm^{-1}. In another study, salt tolerant *Pseudomonas* strain 002 was explored to improve root formation in maize by ameliorating the adverse effect of salinity stress (150 mM NaCl) (Zerrouk et al., 2016). Therefore, it is postulated that application of such indigenous ST-PGPR strains in development of bio-inoculants will not only enhance the crop productivity in saline soils but will also help in achieving food security in the future.

Plant growth-promoting bacterial endophytes (PGPE) are the microorganisms having the ability to occupy plant's interior and establish a special type of symbiotic relationship with the plants where both partners may derive benefits (Reiter and Sessitsch, 2006; Rashid et al., 2012). Endophytic strains colonizes different plant tissues, some primarily inhabit roots of the plants, while others colonize stems and leaves. Estimates suggest that the planet contains about 300,000 plant species and approximately most of the plants accommodate endophytes interior their tissues (Smith et al., 2008; Santoyo et al., 2016). However, metabolic and physiological properties of endophytic microbiota residing in plants have been largely unexplored as successfully cultured endophytic library signifies only a fraction of the whole bacterial community (Walitang et al., 2017). Bacterial endophytes promotes the plant growth as a consequence of phosphate solubilization activity (Wakelin et al., 2004), fixation of N (Compant et al., 2005), phytohormones production (Ratnaweera and Silva, 2017), synthesis of siderophores (Lodewyckx et al., 2002), 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Sun et al., 2009) and supply of indispensable nutrients to the host plants (Puente et al., 2009). ACC deaminase enzyme cleaves ACC to α-ketobutyrate and ammonia and further decreases ethylene levels in host plants. Ethylene is regarded as an important plant growth regulating phytohormone functioning in processes of root initiation, flower wilting, fruit ripening, seed germination, leaf abscission, biosynthesis of other phytohormones, and stress signaling. Plants normally produces only small amounts of ethylene; however, in response to various stresses (flooding, drought, salinity, temperature extremes and nutritional stress) significant rise in ethylene biosynthesis has been observed. In the same regard, PGPE exhibiting ACC deaminase activity may be the best choice in helping plants to keep the stress ethylene concentration below the growth inhibitory point of the particular stress (Glick, 1995; Ali et al., 2014; Khan et al., 2020). Endophytes have been reported to be endowed with the capability to alleviate the adverse effects of salinity and stimulate stress resilience in plants (Kearl et al., 2019; Khan et al., 2020). Furthermore, this endophytic symbiotic relationship also assists in regulating the uptake of mineral nutrients and exudation of defensive metabolites from the roots, and maintaining a balance in plant phytohormones (Bashan et al., 2013; Khan et al., 2017a). Endophytic bacterial residents of rice roots were studied using metagenomics and the sequences obtained from endophytic cell extracts by Sessitsch et al., (2012). The role of metabolic processes such as detoxification of ROS and quorum sensing in improving plant stress resistance were also unfolded in the same study. Therefore, greater efforts must be devoted concerning towards intensification of crop productivity under such stressful conditions with the aid of PGPE which will make salt stressed soil arable to meet the food demands of our ever-growing population.

2.4 Wheat

India is a fast-growing country located to the south-east of the Eurasian countries and its population is growing by 2% a year (Tripathi and Mishra 2017). Agriculture with its allied sectors is unquestionably the largest and primary source of livelihood for the country's population. After independence India's progress in the agricultural sector has led the country to become self-sustaining in its major staple crops particularly rice and wheat (Thorburn, 2015). Wheat (*Triticum* spp.) is a widely cultivated significant cereal crop in terms of production and consumption that occupies the prime position among the 'big three' food crops in the world and has played a vital role in sustaining the food grain production in the country over the past few years (Shewry 2009; Rosenblueth et al. 2018). The plant is an annual grass that grows between ½ to 1 ¼ meter in height, with a long stalk that terminates into a tightly formed cluster of kernels enclosed by a beard of bristly spikes (Smith et al., 2010). Arzani and Ashraf, (2017) stated that the staple food crop provides calories to approximately 30% of the world population (4.5 billion) and fulfils upto 20% of the total protein requirement in the human diet. Since the dawn of civilization, human beings have been known to cultivate, prepare and consume wheat. In the simplest of terms, it is widely consumed especially because of gluten, the main protein of wheat. Gluten is a kind of rubbery substance that is formed when the endosperm of wheat is mixed with water. Wheat plays a big role in the production of bread, biscuits, feeds, confectionery, and various commercial products (Oyewole 2016). It is widely being utilized for human food and livestock feed and is considered as the second important crop next to rice in India (Ramadas et al., 2019). The breeding and domestication of modern wheat have constricted its genetic pool, leaving it susceptible to environmental stress (Peng et al., 2011). The grass undoubtedly serves as one of the predominant basis of human nutrition worldwide although to increase its productivity in the face of climatic change, soil stresses, natural resources depletion, and high food prices lots of major research efforts are still needed.

2.4.1 Production of wheat in India

Wheat is a major rabi crop and is cultivated in the winter season after the reaping of summer crops. The cereal crop is usually sown in the months of October-November and harvested around April. Soil moisture, low temperatures, and well distributed rainfall are some significant prospects that support higher planting and productivity. The nutritionally rich cereal occupies around 217 million hectares globally and the crop is known to grow in variegated environments with an annual production drifting around 731 million tonnes (USDA, 2018). India is the second-largest producer of wheat worldwide as the country has been blessed with the diversified agroecological conditions (Fig. 8). Having a significant share in consumption of the total food grains produced, the wheat crop has been under cultivated in India with an approximate output record of 107.59 million tonnes (MoA&FW 2020). The country was a net deficit in food production after independence and imported wheat for domestic consumption. Afterward, during 1966–1967 various coordinated research and food security-based programs were embraced, which led to the green revolution in India. These revolutionary movements intensified agricultural productivity to meet urgent public needs and also contributed significantly to the commercialization of crop products by enhancing plant productivity and their yields (Ramadas et al., 2020). All India Coordinated Wheat Improvement Project (AICWIP) is one of the largest crop improving network projects that got implemented in 1965 by the Indian Agricultural Research Institute (IARI), New Delhi (Singh et al., 2019; Fig. 9; Table 1). Under this project, more than 498 varieties have been developed so far, which also became extensively popular among farming communities because of their suitability to different agro-ecological conditions. For instance, C 306, Lok 1, Sonalika, Kalyansona, PBW 343, UP 2338, WH 542, GW 322, HD 2967, HD 3086, WB 2 are some of the landmark wheat varieties that were cultivated and became the popular deliverables of the scheme.

Remarkable advancements have been made over the years but still, the problems in northern India such as flood irrigation causing soil salinity, declining water tables, and over-exploitation of groundwater could force the farmers to redirect to less water-intensive crops and is a matter of considerable concern. With increase in human population, the pressure for increasing food production has escalated and also compelled people to introduce high yielding crop varieties in agricultural practices (Raza et al., 2019). Introduction of such high yielding crop varieties lead to increased use of chemical fertilizers. The prolonged use of chemical fertilizers in agroecosystems adversely affect the soil health and causes soil organic matter degradation, soil acidity, and environmental pollution (Kumar et al., 2019; Pahalvi et al., 2021). There is an absolute requirement for N for plant growth, and the crop yield and productivity highly depend upon substantial N inputs (Hawkesford, 2014). Plants also require N for storage proteins in the grain which is one of the important quality attributes of cereal crops. Application of N to the plants in the form of fertilizers increases greenness of plants, crop quality-yield, CO_2 assimilation rate as well as improves resistance to environmental stresses. Hou et al., (2012) stated that nitrogen application is more important in comparison to other major essential nutrient for successful crop production. The demand of N fertilizers has markedly increased since the middle of the 20th century due to the impact of the ‘green revolution’. Urea has highest nitrogen content (46%) of all solid nitrogenous fertilizers and is considered to be one of the most used synthetic nitrogen fertilizers globally (Anas et al., 2020). However, once the nitrogen fertilizers are applied to agricultural systems, they are absorbed directly by plants or converted into various other forms which causes increment in phytoavailability of the nitrogen pool. Consumption of nitrogen fertilizers in excess intensify the potential threat to the surrounding environment for instance eutrophication, the greenhouse effect, and acid rain (Wang et al., 2011).

2.4.2 Salinity and effect of salinization on cereal crop wheat

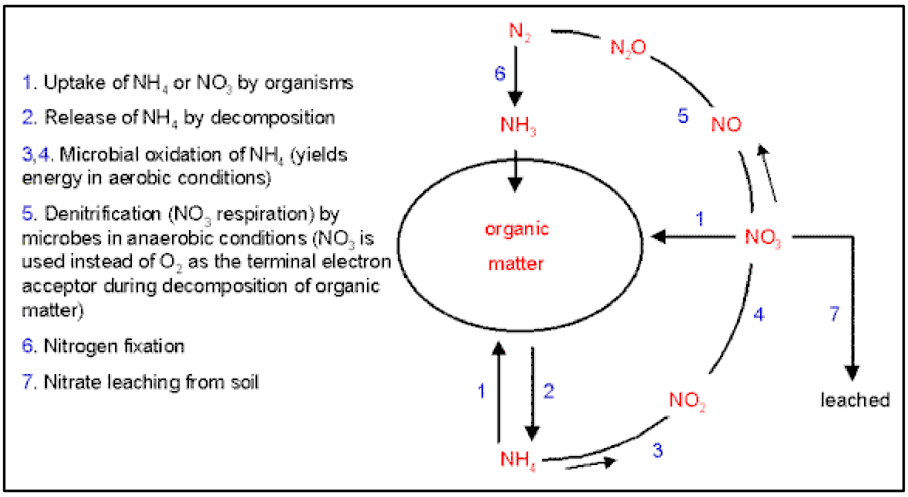
Abiotic stress comprises of any environmental conditions or combination of them which negatively affects the expression of genetic potential for development, growth and reproduction of the wheat plants (Jones and Qualset, 1984; Abhinandan et al., 2018). Salts naturally occur within soils and water. Soil in which water-soluble salts go beyond 4 dS m^{-1} (equivalent to about 40mM NaCl) is considered saline. The process of increase in the salt content is known as salinization and soil salinity is one of the preeminent abiotic stress that tremendously affects the growth, production, and quality of agricultural crops in arid and semi-arid regions worldwide (Tester and Davenport 2003; Ondrasek et al., 2011; Kakar et al., 2019; Hussain et al., 2013). Increment in crop productivity is closely related to irrigation; however, the methods used in irrigation practices for the plants often results into increased soil salinity (Manik et al., 2019). The presence of excess salts in soil occupies a prominent place among the soil problems and has remarkably impacted the environment, ecology, and agriculture in terms of uncertain, unstable livelihood and poor quality of life. It has rendered significant masses of less productive or unproductive land that are projected to increase in the future due to modern climate change scenarios viz. rise in temperature, shrinking glaciers, increase in evaporation, rising sea levels and impact on coastal areas (Arora et al., 2012; Kumar and Sharma 2020). Increment of soluble salts in soil can be natural (primary soil salinity) or maybe anthropogenic due to environmental pollution (secondary soil salinity) (Waters et al., 2013). It has been estimated that out of the world’s 5.2 billion ha of dryland agriculture, 3.6 billion ha is embraced by the problems of erosion, soil degradation, and salinity (Riadh et al., 2010). Data also shows that nearly 147 million ha of land is predicted to be exposed to soil degradation in India, out of which 23 million ha is only subjected to the threat of salt stress/salinity/alkalinity/acidification. The largest area under saline soils (71.2%) is reported to come in the state of Uttar Pradesh (Kumar and Sharma, 2020).

Massive urbanization is putting a lot of pressure on agricultural lands and this growing trend of unproductive land is becoming a threat to national food security and economic development by affecting the growth and yield of diverse crops, including cereal crops.

Wheat is considered to be one of the moderately salt-tolerant crops but its yield starts to decline at high saline concentrations i.e., 6-8 dS m^{-1} (Royo and Abió 2003). Marginal environments comprise of any environmental conditions which negatively influences the expression of genetic potential for plant growth and its development (Akbarimoghaddam et al., 2011). The productivity of wheat is often severely affected by salinity stress which is associated with reduced growth, decreased germination percentage, disrupted photosynthesis, altered enzymatic activity, altered reproductive behavior, imbalance in hormone levels, and oxidative stress (Hasanuzzaman et al., 2014; Çelik and Atak 2012). At 8.8 dS/m the plants of wheat emerged decrease to 50 percent (Francois et al., 1986). High content of NaCl influences the metabolic processes in the plant by deteriorating the water potential of cells, mineral nutrients uptake, membrane integrity, and ion toxicity (hyperosmotic and hyperionic) (Fig. 10). Reactive oxygen species (ROS) are also formed due to water deficiency in salt stressed environments that may induce phytotoxic reactions such as degradation of chlorophyll, membrane-lipid peroxidation, and DNA mutation in the plants. Na+ is the principal toxic ion responsible for imposing both osmotic as well as ionic toxicity in the plants and thereby maintaining its level inside the cytoplasm is the only mechanism plants may adapt to survive under salt stress environments (Erdmann and

Hagemann 2001; Fukuda et al., 2004; Anil et al., 2007). Excess of Na+ also inhibits the uptake of essential macronutrients like Ca2+ and K+ from soil and this highly imparts negative effects on plant nutrition. Significant variations in the tolerance level of salinity have been observed in different wheat germplasm. Alarmingly, the unceasing expansion of salt affected land poses a severe threat to sustainable agriculture and become a major cause of concern in few of the economically challenged and highly populated countries for instance India (7 million hectares (Mha); Vashev et al., 2010), Bangladesh (1 Mha; Hossain, 2010), Pakistan (6 Mha; Qureshi et al., 2008; Mujeeb-Kazi et al., 2019). Considering the detrimental effects of salt stress in wheat crop, several researchers are working on possible solutions and trying to survey salt-tolerant strategies in plants by different approaches. Plant tolerance to salinity is a complex phenomenon and involves the use of osmoprotectants, plant hormones, molecular markers, QTL, application of plant nutrients, development of improved transgenic wheat cultivars. Despite intensive efforts and the availability of reports on wheat and salinity tolerance, our knowledge still seems to be limited in terms of productively improving wheat growth and development in saline conditions. Therefore, it is the need of the hour that we should recline ahead for some combined approaches that can assist in finding new paradigms to improve crop productivity with respect to salinity stress in the near future.

It is strongly needed to put greater attention to the studies pertaining to the identification and characterization of endophytes in respect to their impact on the mitigation of abiotic stresses in the crops. We should also look forward to new strategies that support microbe-mediated based stress mitigation in the future and for the same more focused omics-based studies encompassing genomics, metagenomics, proteomics, and metabolomics are highly needed.



1. Uptake of NH_4 or NO_3 by organisms
2. Release of NH_4 by decomposition

3,4. Microbial oxidation of NH_4 (yields energy in aerobic conditions)

5. Denitrification (NO_3 respiration) by microbes in anaerobic conditions (NO_3 is used instead of O_2 as the terminal electron acceptor during decomposition of organic matter)
6. Nitrogen fixation
7. Nitrate leaching from soil

Fig. 5 Overview of the nitrogen cycle in soil or aquatic environments

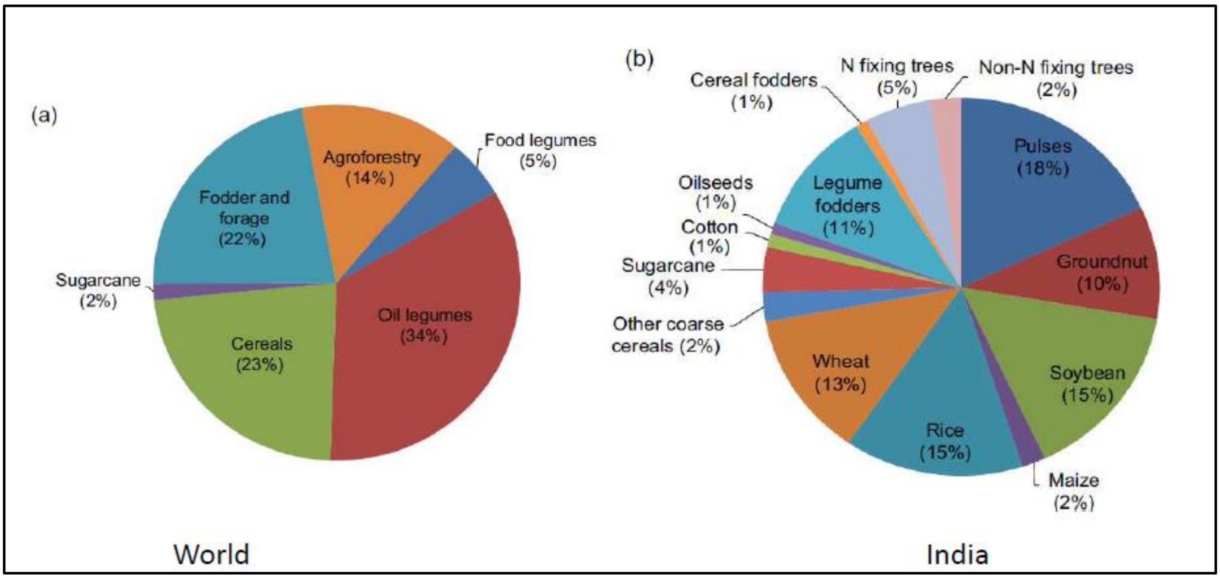


Fig. 6 Annual biological nitrogen fixation in agriculture (Rao and Balachandar, 2017)

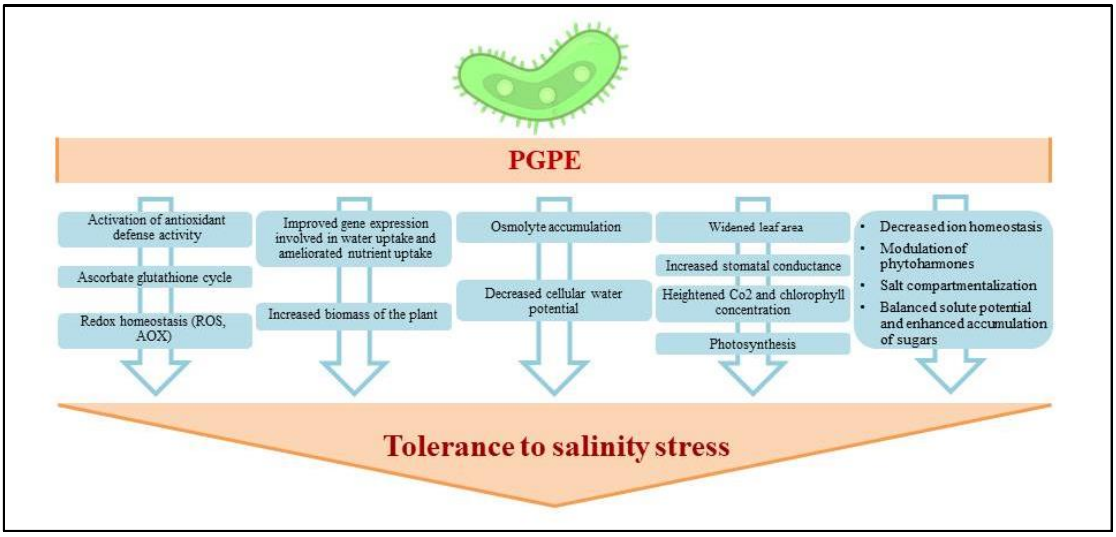


Fig. 7 Scheme summarizing the mechanisms deployed by plant growth promoting endophytes (PGPEs) to alleviate abiotic salt stresses

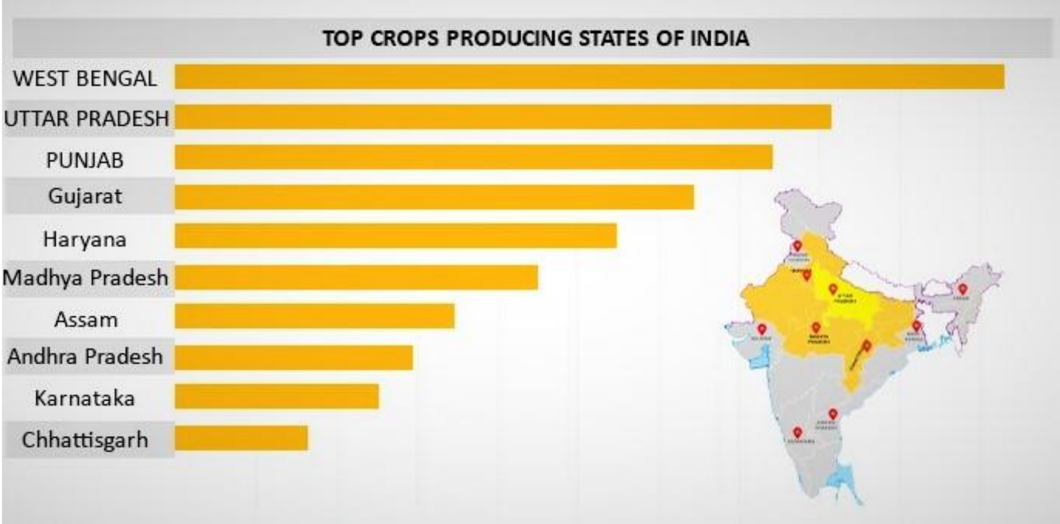
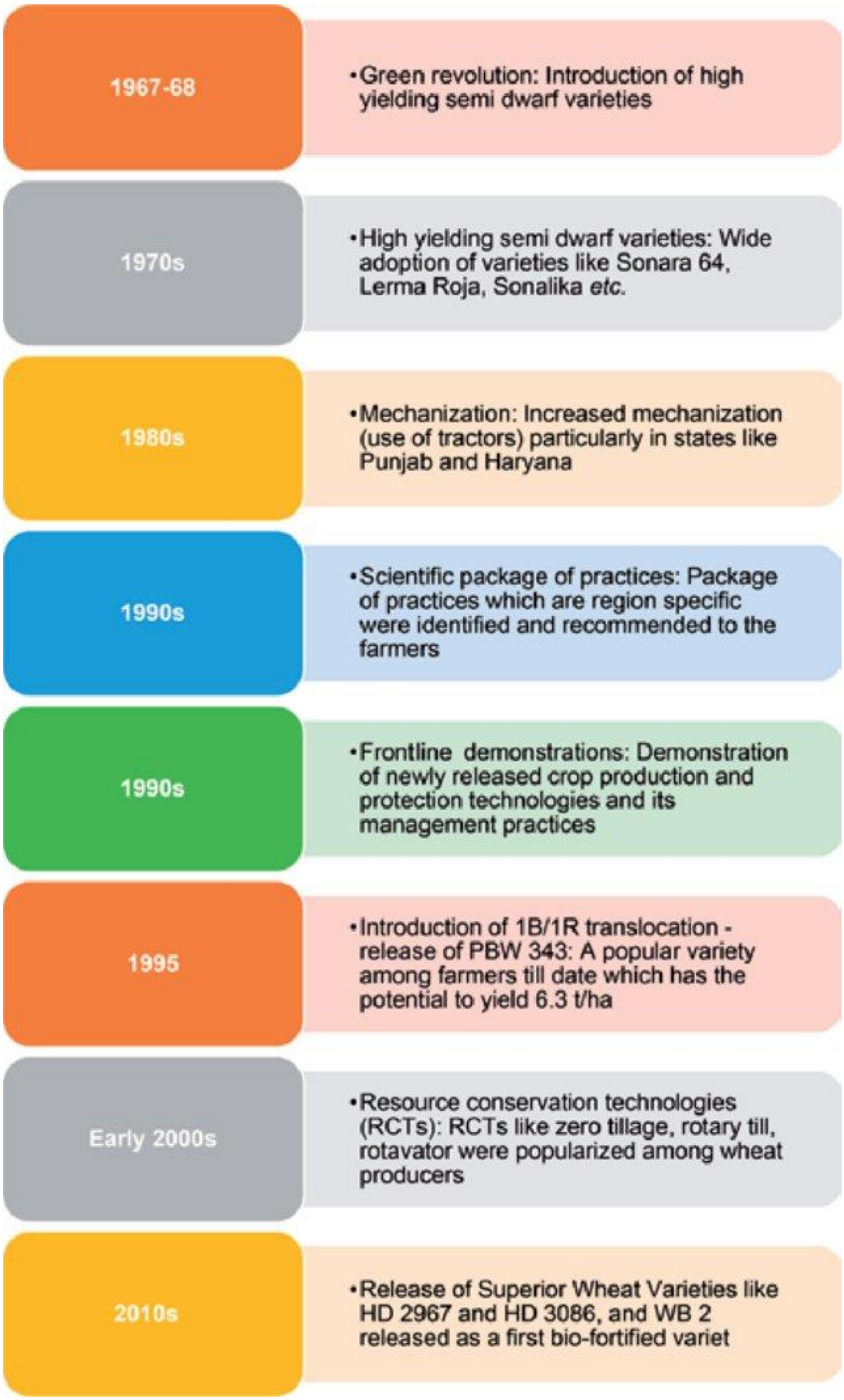


Fig. 8 Top ten Wheat Producing States in India (Source: https://krishijagran.com/blog/top-10-leading-agriculture-states-of-india-with-most-crops/)



1967-68	•Green revolution: Introduction of high yielding semi dwarf varieties
1970s	•High yielding semi dwarf varieties: Wide adoption of varieties like Sonara 64, Lerma Roja, Sonalika *etc.*
1980s	•Mechanization: Increased mechanization (use of tractors) particularly in states like Punjab and Haryana
1990s	•Scientific package of practices: Package of practices which are region specific were identified and recommended to the farmers
1990s	•Frontline demonstrations: Demonstration of newly released crop production and protection technologies and its management practices
1995	•Introduction of 1B/1R translocation - release of PBW 343: A popular variety among farmers till date which has the potential to yield 6.3 t/ha
Early 2000s	•Resource conservation technologies (RCTs): RCTs like zero tillage, rotary till, rotavator were popularized among wheat producers
2010s	•Release of Superior Wheat Varieties like HD 2967 and HD 3086, and WB 2 released as a first bio-fortified variet

Fig. 9 Major developments in India post inception of the All India Coordinated Research Project (Ramadas et al., 2019)

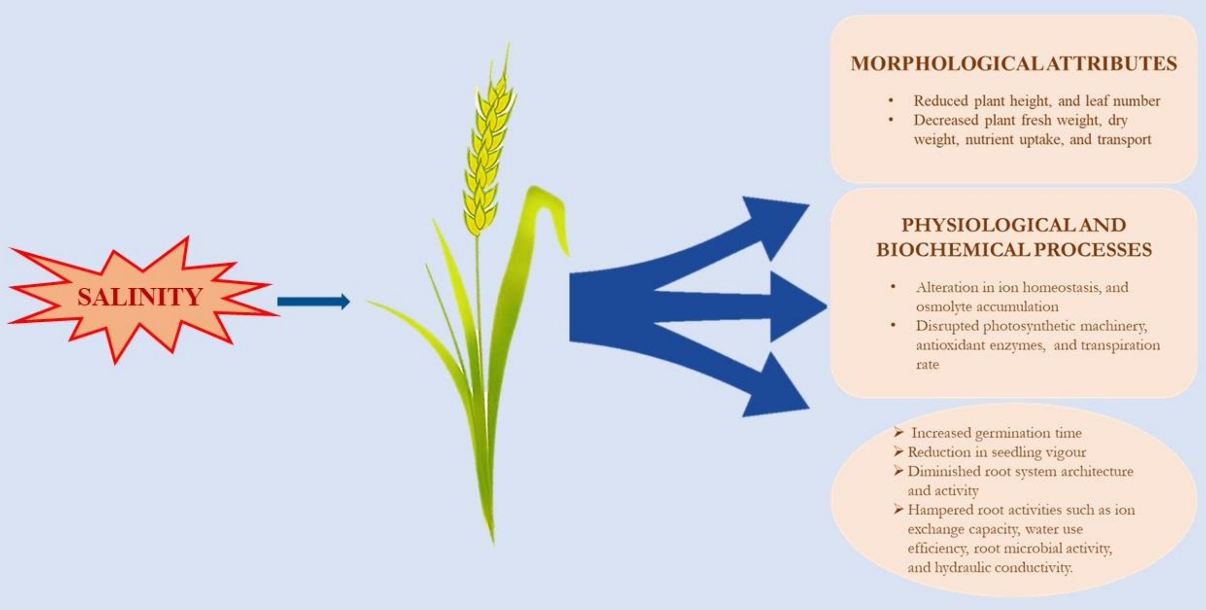


Fig. 10 Salinity stress on different growth stages of wheat plant

Table. 1 Data showing quinquennial average of area and production of wheat (kg/ha) (modified from Ramadas et al., 2019)

S.No.	**State/UT**	**Area (million ha)**	**Production (million tonnes)**
		2013–2014 to 2017–2018	**2013–2014 to 2017–2018**
1.	Assam	0.02	0.03
2.	Bihar	2.08	4.86
3.	Chhattisgarh	0.10	0.14
4.	Gujarat	1.09	3.22
5.	Haryana	2.55	11.24
6.	Himachal Pradesh	0.34	0.66
7.	Jammu and Kashmir	0.29	0.48
8.	Jharkhand	0.19	0.38
9.	Karnataka	0.19	0.20
10.	Madhya Pradesh	5.73	16.32
11.	Maharashtra	1.05	1.48
12.	Punjab	3.51	16.61
13.	Rajasthan	2.98	9.31
14.	**Uttar Pradesh**	**9.75**	**27.93**
15.	Uttarakhand	0.34	0.81
16.	West Bengal	0.29	0.80
17.	Others	0.04	0.12
18.	India	30.54	94.57

MATERIALS

&

METHODS

3.1 Sample collection

In this study, the endophytic bacteria were isolated from healthy roots of wheat (*Triticum aestivum* L.) which were procured from central regions of Uttar Pradesh, India. Plant samples were randomly collected from salt affected and assorted areas of central regions of Uttar Pradesh i.e., Lucknow (26.8467° N, 80.9462° E), Kanpur (26.4499° N, 80.3319° E) Unnao (26.5393° N, 80.4878° E) and Raebareli (26°15.5 N 81°28.9 E). The details of the sampling sites along with their geographical location have been outlined in Table 2. Intact wheat plants along with healthy root system were carefully uprooted and roots were segmented for further isolation of endophytic bacterial isolates.

3.2 Enrichment and isolation of bacterial endophytes

Procured roots were washed with running tap water followed by distilled water until adhered soil was removed completely. They were then detached from the plant very carefully and sectioned into small pieces. Further root samples were surface sterilized to eradicate epiphytic microbes by immersing in 70% alcohol for 30 seconds and sodium hypochlorite for 5 minutes and 0.1% mercuric chloride solution for 5 minutes (Hallmann et al. 2006). To remove the effect of disinfectants, root sections were rinsed six times with autoclaved distilled water. To confirm the sterility of the roots sample after disinfection, 100 µL of water was taken from the final wash and plated on nutrient agar. The inoculated plates were incubated at 29^{o}C for 48 to 72 hours and microbial growth was observed. Root tissues were crushed using a glass rod in a sterile mortar and pestle containing one ml of distilled water. Serial dilutions were made for each macerated root tissue extract and 0.1 ml aliquot from different dilutions (up to 10^{-5}) was spread onto nutrient agar and yeast extract mannitol agar (YEMA) medium plates. For each dilution, the plating was conducted in triplicates. After incubation, the bacterial isolates were observed for their growth, and afterward phenotypically distinguished colonies were purified and sustained as pure cultures. All endophytic isolates were further maintained in 25% glycerol culture stocks at -80°C for long-term storage.

3.3 Internal structure of roots

Analysis of the internal structure of root tissues of wheat was done by microscopy. Fresh root samples were taken and cut into very thin slices. The sections were then observed under the light microscope as well as scanning electron microscope (Model: JEOL, JSM 6490LV) at University Sophisticated Instrumentation Unit (USIC) BBA University, Lucknow.

3.4 Characterization of endophytic isolates

The isolated bacterial endophytes were individually characterized as per the standard protocols of Bergey's Manual of Systematic Bacteriology (Garrity et al., 2005).

3.4.1 Morphological and physiological characterization

i. Colony morphology

The growth of isolated endophytes was monitored on different nutrient medium plates. The colonies were observed for their growth pattern, shape, size, color, elevations, and texture (Vincent 1970).

ii. Gram staining

Morphological differences in the structure of bacterial cells were analyzed by Gram staining and performed according to Gram (1884). A loopful of culture to be examined was taken and aseptically transferred on a slide containing a drop of water. The bacterial smear of each isolate was prepared with the help of an inoculation loop. The smear was stained by flooding crystal violet for 60 seconds and then rinsed with distilled water. Afterward, a few drops of Gram's iodine was put on the smear for 60 seconds, washed with 95% alcohol for about 10-20 seconds until the purple dye no longer flows from the smear, and again gently rinsed with water. In last the smear was flooded by a counterstain safranin for about 1 minute and washed with distilled water. The slide was air-dried and was observed under the microscope to determine the shape and Gram's nature of bacterial isolates.

Cell morphology of the isolates was also studied by analyzing them under a scanning electron microscope (JEOL, JSM-6490LV) at USIC, BBA University, Lucknow.

iii. Generation time

Growth profile study was done and the generation time of endophytic isolates was calculated. 100 ml of sterilized nutrient broth was prepared and inoculated with loopful log phase culture. After incubating the samples at ambient temperature, the bacterial growth of each isolate was assessed by measuring the optical density after every 4 hours in a UV-VIS spectrophotometer (Thermo Scientific, Evolution 201) (Dubey and Maheshwari 2012) at 600 nm. Generation time was calculated using the formula:

$$\text{Generation time} = (T2 – T1) / 3.3 (\log_{10} OD2 - \log_{10} OD1)$$

Where (T2-T1) stands for the difference of two-time intervals at any two-point in log phase in growth curve; and (log10 OD2- log10 OD1) stands for the difference between the $\log_{10}$ value of OD2 at time T2 and OD1 at time T1.

iv. Motility

This test was performed to examine whether procured isolates were motile or not and it was done by using a semisolid agar medium (Tittsler and Sandholzer 1936). The microorganisms were stabbed into the motility test medium with the help of a needle. The tubes were further kept for incubation at 28°C for 24 hours and were observed for the growth pattern of the isolates. Motility of the bacterial isolates was also assayed by hanging drop method (Ogale et al., 2018).

v. Growth at different pH

The tolerance of the selected bacterial endophytic isolates to pH was studied according to Zablotowicz and Focht (1981), by varying the pH range from 5.0 to 12. NA was used to determine the tolerance ability and pH was accustomed by addition of HCl or NaOH. The inoculated (using log phase culture of 10^8 cfu/ml) medium was incubated for 25 ± 2°C for 3-5 days and observed for bacterial growth.

vi. Salt tolerance assay

Halotolerance assay of the selected endophytic isolates was performed by inoculating NB medium amended with various salt concentrations ranging from 2 to 10% NaCl (w/v). The log phase bacterial culture of the endophytic isolates (OD 610 adjusted to 0.1) were inoculated to the broth medium and incubated at 25 ± 2°C for 48 h at 120 rpm. Optical density (at 610 nm) was measured using a spectrophotometer (Thermo Scientific™ Evolution 201 UV–vis) after every four hours up to stationary phase and salt-tolerance growth kinetics curve was drawn (Khare et al. 2011). Medium without NaCl was considered as non-saline control and the experiment was done in triplicates.

vii. Utilization of different carbon sources

The endophytic isolates were checked for their ability to utilize different carbon sources like glucose, dextrose, lactose, galactose, sucrose and maltose. The mannitol was replaced by other sources of carbon from Yeast extract mannitol agar (YEMA) medium (Zablotowicz and Focht 1981). Each carbon source was added to medium in the final concentration of 10% (w/v) after sterilization (filtered through Millipore membranes of pore size 0.22 μm). Isolates were streaked and observed for growth after incubation at 28 °C for 24 to 48 hrs.

viii. Utilization of different nitrogen sources

Ability of isolates to survive on various nitrogen sources was checked on YEMA by replacing yeast extract with various nitrogen sources such as yeast extract, potassium nitrate, sodium nitrate, ammonium chloride, ammonium sulphate, and tryptophan (Holt et al. 1994). Medium plates were prepared, autoclaved, and inoculated with loopful of bacterial culture. After incubation at 28 ºC for 24 to 48 hrs, growth of all the isolates were observed.

ix. BTB test

Bromothymol blue (BTB) was used in order to determine whether the isolates are fast growers or slow growers (Somasegaran and Hoben 1994; Norris 1965). Nutrient agar plates supplemented with indicator dye 0.25mg/L of BTB were inoculated with the bacterial endophytic culture and incubated at 28±2ºC for 2-10 days. The color change reaction of the medium from green to yellow indicated acid and green to blue indicated alkali production.

3.4.2 Biochemical characterization of endophytic isolates

i. Indole production

Loopful of test organisms were inoculated in tryptone broth and incubated for 24 hrs at 28 ºC (Cheesbrough 1985). 1 ml of Kovac's reagent was added to each test tube after incubation. A positive test was indicated by the formation of a red color ring on the surface layer of broth.

ii. Ammonia Production

The ability of endophytic bacterial strains to produce ammonia was qualitatively detected by the protocol given by Cappuccino and Sherman (1992). 5 ml of peptone broth was dispensed in separate tubes and was inoculated by bacterial endophytic isolates. Inoculated tubes were incubated for 48-72 h at 28ºC. 1 ml of Nessler's reagent was added in each test tube and color change of broth from slightly yellow to orange and brown was observed.

iii. Catalase test

The catalase activity of isolates was determined by placing a loopful of fresh bacterial culture on a clean glass slide. A few drops of 3% hydrogen peroxide (H_2O_2) were poured onto the microscopic slides and were further observed for effervescence (Graham and Parker 1964). The appearance of gas bubbles of oxygen indicated the activity of the catalase enzyme. Motility of bacteria was also observed by hanging drop method according to Bertrand et al. (2001).

iv. Citrate utilization test

The endophytic bacteria were assayed for citrate utilization on Simmon's citrate agar plates. Plates of the medium were prepared and spot inoculated with bacterial cultures. The medium plates including one uninoculated control were kept for incubation at 28 ºC for 3-5 days (MacWilliams 2009). Appearance of color change from green to blue was observed.

v. Methyl Red and Voges Proskauer (MR-VP) test

Methyl Red and Voges Proskauer (MR-VP) broth was prepared and sterilized. 10-10 ml was transferred in distinct tubes and the tubes were inoculated by a loopful culture of endophytic isolates (Mac Faddin and Jean 1976). Test tubes containing broth were placed for incubation at 28 ºC for 48 h and after incubation broth from each test tube were equally divided and two sets were made. To the first set of tubes, 5 drops of methyl red were added and tubes were observed for distinct red color that was an indicator of positive reaction for MR test. 12-15 drops of VP- I reagent (5% α-naphthol solution in 70% ethyl alcohol) and VP- II reagent (40% potassium hydroxide) was added for VP test to another second set of test tubes and shaken gently. The sample was kept for 10-15 minutes and development of red color was considered as a positive result for the VP test.

vi. Nitrate reductase activity

Endophytes were cultured on tryptone yeast extract (TYE) medium that contained 0.1% KNO_3 and incubated in the absence of light for 24-48 hrs at 25°C (Campbell 1999). Few drops of sulphanilic acid (8 g/l in 5M acetic acid) and solution of α-naphthylamine (5g/l in 5M acetic acid) were then added to each tube. Pink color of the resultant solution was observed that denoted nitrite formation.

vii. Urease test

Urea agar plates were prepared in which phenol red was added as an indicator dye. The medium was autoclaved and slants were prepared. Each bacterial isolates along with control were streaked on agar surface followed by incubation for 24 to 48 hrs at 28 ºC (Lindström and Lehtomäki 1988). Change in color of the medium from yellow to pink designated production of urease.

viii. Gelatinase production

In this test, gelatin agar medium was made and poured in test tubes, and in all the tubes stab inoculation of each isolate was made separately (Chapman 1952). Tubes were incubated at 28ºC for 5-6 days. They were then transferred to the refrigerator at 4ºC for 15 minutes and were further observed for partial or total liquid state of gelatin.

ix. Amylase test

Starch agar was prepared and the medium plates were spot inoculated with the endophytic isolates (Lennette, 1985). After incubation for 48 hrs, Gram’s iodine solution was flooded onto the medium plates. Clear and transparent zone were noticed after the removal of extra iodine solution.

x. Lipase test

Lipase activity test was performed on Tween 80 agar media following the protocol of Samad et al. (1989). The isolates were inoculated into media plates and were incubated for 3-4 days at 28 ºC. After the incubation period, a clear opaque zone around the colony was observed which pointed towards the presence of lipase enzyme.

xi. Protease test

Each bacterial isolate was assessed for their proteolytic activity by inoculating them on skimmed milk agar (SMA) media plates and incubated for 4-5 days at 28°C (Kasana et al. 2011). Clear zone was noted around the active bacterial colonies.

3.5 Plant growth promoting characterization

3.5.1 Siderophore production

The ability of the isolates to produce siderophore was checked on the Chrome azurol Sulphonate (CAS) agar medium by universal CAS assay (Schwyn and Neilands, 1987). To get rid of trace elements of iron the glasswares were initially cleansed by 3M hydrochloric acid (HCl) and afterward finally rinsed with deionized water. Siderophore production was anticipated qualitatively as well as quantitatively. Preparation of CAS reagent was done by dissolving 121 mg CAS dye in 100 ml distilled water. Afterward, a separate solution was made in which 20 ml of 1 mM ferric chloride ($FeCl_36H2O$) solution was added in 10 mM HCl. Both the solutions were mixed and gradually added to 20 ml hexadecyl trimethyl ammonium bromide (HDTMA) solution (729 mg HDTMA in 400 ml distilled water). This resultant mixture was constantly stirred followed by sterilization and is known as CAS-HDTMA solution.

i. **Qualitative assay:** The test was performed according to the modified protocol by Hu and Xu (2011). Plates of CAS agar medium were prepared by mixing 100 ml CAS reagent in 900 ml of LB agar medium (sterilized before use). Isolates were spot inoculated on each plate and were incubated for 5-7 days at 28ºC. An uninoculated plate was taken and considered as the experimental control. Development of halo zone with color change from blue to orange around the bacterial colonies was considered as positive for siderophore production.

ii. **Quantitative assay:** Production of siderophore was also quantitatively estimated as per CAS-Shuttle assay (Arora and Verma, 2017). For the same, the cultures of bacterial isolates were grown in LB broth medium at 28ºC for 4-5 days and were centrifuged (10,000 rpm for 10 min). The supernatant was used to estimate the siderophore production using spectrophotometric analysis. 0.5ml of culture supernatant was transferred to a fresh test tube and was mixed with an equal amount (0.5 ml) of CAS reagent and uninoculated broth mixed with CAS reagent was used as blank. OD was measured after 20 min at 630 nm in a spectrophotometer (ThermoScientific, Evolution 201). The amount of siderophore produced by strains was expressed in percent siderophore unit (psu) and calculated according to the formula:

$$\text{Siderophore production (psu)} = \frac{(Ar-As) \times 100}{Ar}$$

Where, Ar = Absorbance of blank (CAS solution and un-inoculated broth)

As = Absorbance of the sample (CAS solution and cell free supernatant of sample)

3.5.2 Indole-3-Acetic Acid (IAA) production

The ability of endophytic isolates to produce IAA was examined by growing the cultures in a nutrient broth medium supplemented with 0.5% (w/v) L-tryptophan. The tubes were incubated for 4 days at 28ºC and the cultures were centrifuged at 5000 rpm for 15 min in order to remove the bacterial cells. Subsequently, 1 ml of supernatant was collected in a separate test tube and 2 ml of Salkowski's reagent solution (1.0 ml of 0.5 M FeCl3, 30 ml of concentrated sulfuric acid, and 50 ml of distilled water) was added. The resultant was thoroughly mixed and stored for 30 minutes at 28°C in a dark place. Appearance of red/pink color indicating presence of IAA in the reaction mixture was then observed (Salkowski 1885). Quantitative estimation of IAA production was also carried out by measuring the absorbance of each sample at 530 nm by spectrophotometer (Thermo Scientific, Evolution 201). A standard curve of pure IAA of 0.5-100 µg/ml concentration was also plotted and the amount of IAA produced (µg/ml) was determined.

3.5.3 Phosphate solubilization

Phosphate solubilization assay was done by spotting the isolated endophytes on Pikovskaya’s (PVK) agar media containing tricalcium phosphate. The inoculated plates were kept for incubation at 28 ± 2°C for 5 days (Pikovskaya 1948). Formation of clear halo zone around the endophytic colonies was observed and phosphate solubilization index (PSI) was calculated (Premono et al., 1996) by using the formula:

$$\text{PSI} = \frac{\text{Colony diameter + halo zone diameter}}{\text{Colony diameter}}$$

3.5.4 Zinc solubilization

All isolated endophytes were screened for their zinc solubilization potential following the method of Fasim et al. (2002). Spot inoculation was done on Zn solubilizing basal agar medium supplemented with 0.1% of zinc oxide (ZnO) and zinc carbonate ($ZnCO_3$). The_plates were covered with aluminum foil and incubated in the dark for 5-6 days at 28°C. Clear zones around the grown colonies were observed and the diameter of these transparent zones was recorded. Zinc solubilization index (ZSI) was calculated by:

$$\text{ZSI} = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

3.5.5 Hydrogen cyanide (HCN) production

Qualitative determination of hydrogen cyanide (HCN) was carried out by streaking the endophytic isolates on a nutrient agar medium supplemented with 4.4 gl-1 of glycine (Bakker and Schippers, 1987). Each bacterial culture was streaked on a separate petri plate and the uninoculated plate was used as a test control. The production of cyanide was detected by placing Whatman No.1 filter paper soaked in picric acid (0.05% solution in 2% sodium carbonate) in the lid of the petri plates. The plates were sealed air-tight with parafilm and incubated at 30°C for 6-7 days. Development of brown to red color after incubation was observed which indicated HCN production.

3.5.6 Potassium solubilization

The assay was performed on modified Aleksandrov agar medium by spot test method under aseptic conditions (Parmar and Sindhu 2013). Plates of modified Aleksandrov agar were prepared distinctly i) having mica powder as an insoluble form of K; ii) having K2HPO4 i.e., soluble form of potassium. Spot inoculations of bacterial strains were made onto both types of medium plates and incubated at 28±2°C for 4-5 days. Clear halo zone formation indicated the solubilization of potassium by the isolates.

3.5.7 ACC deaminase assay

The isolates were qualitatively estimated for ACC deaminase activity on the sterile minimal DF salts medium (DF salts per liter: 4.0 g KH2PO4, 6.0 g Na2HPO4, 0.2 g MgSO4.7H2O, 2.0 g glucose, 2.0 g gluconic acid, and 2.0 g citric acid with trace elements: 1 mg FeSO4.7H2O, 10 mg H3BO3, 11.19 mg MnSO4.H2O, 124.6 mg ZnSO4.7H2O, 78.22 mg CuSO4.5H2O, 10 mg MoO3, pH 7.2) amended with 3 mM ACC instead of (NH4)2SO4 as sole nitrogen source (Dworkin and Foster, 1958; Gupta and Pandey, 2019). The inoculated plates were placed for incubation for 3 days at 28°C and the colonies growing on plates were considered ACC deaminase producers.

3.6 Screening of endophytic isolates for nitrogen fixation

3.6.1 Nitrogen fixation

Nitrogen fixation assay was qualitatively done by inoculating the endophytic cultures in nitrogen free Jensen's medium (Jensen, 1942). This particular medium is specifically used for cultivating nitrogen fixing microorganisms. Isolates were streaked onto prepared medium plates and the plates were then incubated at 30^{o}C for around 4-5 days. Growth appearance of bacterial colonies was observed and considered as a positive result.

3.6.2 PCR amplification of nifH

The nitrogen fixation ability of the isolates was also determined by detecting the presence of *nifH* gene in endophytic isolates using polymerase chain reaction (PCR) technique. Each morphotype was subjected for the isolation of genomic DNA and the DNA fragments were then amplified to detect the presence of *nifH* gene. Extraction of DNA was carried out by following the standard protocol of phenol chloroform extraction method. The primers used in the study were: 19F (5'-GCIWTYTAYGGIAARGGIGG-3') and 407R (5'-AAICCRCCRCAIACIACRTC-3'). The conditions maintained were pre-run for 2 min at 95^{o}C, 40 cycles of initial denaturation for 30 sec at 94°C, annealing for 1 min at 50°C, extension for 0.5 min at 72 °C, followed by a post-run for 5 min at 72°C (Ueda et al., 1995). To visualize the amplified PCR product, samples were processed for agarose gel electrophoresis for the separation of DNA fragments and further analysis.

3.6.3 Acetylene reduction assay

Acetylene reduction assay (ARA) was used to quantitatively examine the nitrogen fixation efficiency of the isolates. Freshly grown pure culture (48 h) of bacterial isolates was taken and cell pellets were formed in order to remove the N traces. The obtained pellets were further inoculated into Modified Burk's nitrogen free medium (50 ml) and incubated for 5 days on a rotary shaker at 28^{o}C. Aliquots (0.1 OD at 610 nm) were obtained after incubation till exponential phase; 20 ml was transferred into the assay vials with an air tight lid equipped with a suba seal. The gas in headspace of the vials was replaced with acetylene (10% v/v) and were allowed to incubate for 24 hours at optimum growth temperature After incubation, gas was withdrawn from the vials and injected into a gas chromatograph (Agilent 7890A GC System) fitted with a HP-5 column and FID detector. The oven temperature was adjusted to 45°C and ethylene production was assayed.

3.7 Determination of endophytic nature of selected isolates

3.7.1 Root colonization assay

Endophytic nature of the selected isolates was determined by testing their ability to effectively infect and colonize inside the plantlet tissue (Hernawati et al., 2011). Seeds of wheat (Annapurna PBW-343) were made endophyte-free by treating them with sodium hypochlorite (NaOCl) solution for 5 min and thoroughly washed three times with distilled water. After surface sterilization, the seeds were treated with the freshly grown culture of the endophytic isolate and were allowed to grow on petri dishes containing water agar for 1-2 weeks under aseptic conditions. Experiment was conducted in triplicates. The roots were taken from germinated plants and crushed with the help of sterile mortar and pestle. Bacterial endophytic isolates were re-isolated using the same protocol as described above. The obtained colonies were then processed for molecular identification by 16S rRNA sequencing and were also matched with the lab culture of the selected isolate (Tewari and Sharma 2020; Fatima and Arora 2021).

3.7.2 Scanning electron microscopy

The endophytic-plant association was also assessed by means of scanning electron microscopy (SEM). Specimens were prepared by fixing the root tissues with the help of 2·5% solution of glutaraldehyde in phosphate-buffered saline for 30 min. Samples were treated with ethanol solutions of increasing concentrations for dehydration and after the final step in absolute ethanol, the roots were dissected for cross-sections (Akhdiya et al., 2014). The sectioned samples were then attached to stub specimens with double cello-tape, coated, and examined at various magnifications by using a scanning electron microscope (Model: JEOL, JSM 6490LV) at University Sophisticated Instrumentation Unit (USIC) BBA University, Lucknow.

3.8 Molecular identification by 16S rRNA gene sequencing and phylogenetic analysis

Genotypic characterization of few endophytic bacterial isolates (selected on the basis of PGP traits and salt tolerance) was done by 16s rRNA gene sequencing. Best performing isolates that have been chosen for further study on the basis of promising plant growth promoting traits and their potential to ameliorate salt stress were further identified at species level.

Endophytic isolates were cultured on nutrient agar medium at 28°C for 4-5 days and genomic DNA was isolated following standard protocols. PCR amplification of gene fragment was done by using universal primers: 243F (5′-GGATGAGCCCGCGGCCTA-3′) and 1378R (3’-CGGTGTGTACAAGGCCCGG-5’). The obtained amplified products were then analyzed electrophoretically through 1 % agarose gel stained with ethidium bromide (10 mg/ml) and bands were observed under UV light.

The cycling conditions maintained for the PCR reactions were:

PCR Amplification conditions and primer details

Reaction mixture (100 µl)	
DNA	1 µl
Forward Primer	400 ng
Reverse Primer	400 ng
dNTPs	4 µl
10X Taq DNA polymerase Assay Buffer	10 µl
Taq DNA Polymerase Enzyme	1 µl
Nuclease free water	Volume makeup 100 µl

Cycling Conditions		
Initial Denaturation	3 minutes at 94°C	
Denaturation	1 minute at 94°C	35 Cycles
Annealing	1 minute 55°C	
Extension	2 minutes at 72°C	
Final Extension	7 minutes at 72°C	

S.No.	**Oligo Name**	**Sequence (5`-3`)**	**Tm (°C)**	**GC-Content**
1.	16s Forward	GGATGAGCCCGCGGCCTA	57	72.22%
2.	16s Reverse	CGGTGTGTACAAGGCCCGG	58	68.42%

The gene sequences of endophytic isolates were further processed by using the software Molecular Evolutionary Genetics Analysis (MEGA, ver. 6.0) and analyzed afterward by BLAST through National Center for Biotechnology Information (NCBI)-BLASTn programme (www.ncbi.nlm.nih.gov/BLAST) (Altschul et al. 1990). Phylogenetic tree showing evolutionary history was constructed according to the Neighbor-Joining method.

3.8.1 Sequence submission to NCBI GenBank database

Gene sequences of selected isolates have also been deposited in the GenBank database of NCBI and accession numbers of each isolate were acquired.

3.8.2 Submission of useful endophytic strains in culture collection center

Selected potential endophytic isolates were submitted as type strains in culture collection repository of National Agriculturally Important Microbial Culture Collection (NAIMCC), Mau, India as general deposit. NAIMCC is an international culture collection center approved by International Depository Authority (IDA). Deposited cultures were assigned with the accession number by collection center authorities.

3.9 Pathogenicity test

The characterization of hemolysis properties of endophytic isolates was primarily checked to determine biosafety issues of the isolates on human health. A loopful culture of each single colony was spot inoculated on the surface of the sheep blood agar medium plates and the plates were allowed to incubate at 37°C for 24-48 hours. Incubated plates were further observed for clear zone of haemolysis.

Pathogenicity tests were also performed on agar medium comprising of 2% sucrose, 0.5% yeast extract, 2% peptone, 2% KCl and 1.5% agar (Vermelho et al. 1996). Activities of extracellular proteases including caseinases and gelatinase were checked by using different substrates. Agar medium individually amended with gelatin and casein were prepared and inoculated with loopful of the bacterial cultures. Plates were incubated for 9-10 days at 37°C and clear zones indicating the hydrolysis of substrates were observed. Catalase tests were also performed following the protocol of Graham and Parker (1964) to know the effect of enzyme in neutralizing the bactericidal effects of hydrogen peroxide which indirectly correlates with pathogenicity.

3.10 Application of isolates to assess their impact on the growth of wheat

Seven endophytic isolates were selected on the basis of nitrogen fixation and salt tolerance and were denoted as AU15, CP18, PD22, PD25, KA28, KA31, OC36. These isolates with multiple PGP attributes were further evaluated for their impact on wheat plants in pot culture experiments. The study was conducted for two consecutive years (2019 and 2020) during the rabi season i.e., November-February in earthen pots sized 15 × 11 × 11 cm. The pots were filled with 5 kg of autoclaved saline soil (EC 9.72 dS/m) which was collected from adjoining areas of Lucknow, Uttar Pradesh, India (26° 72 E, 80° 85 N). Wheat seeds of variety Annapurna-PBW 343 were selected for pot culture experiments. The physicochemical properties of experimental soil including pH, electrical conductivity (EC), organic carbon, available N, P, and K were analyzed as per the standard protocols (Jackson, 1973; Chapman and Pratt, 1961) (Table 13). Set of treatments used in the experiments are mentioned below:

1) Untreated control
2) Seeds + AU15
3) Seeds + CP18
4) Seeds + PD22
5) Seeds + PD25
6) Seeds + KA28
7) Seeds + KA31
8) Seeds + OC36

Prior to sowing, the seeds were surface sterilized with NaClO (diluted 1:5 in sterile water) distilled for 5 min; washed with 1% Tween for 5 min followed by thorough rinsing with deionized water (Gamalero et al. 2004; Weller and Cook 1983). Solution of 1% carboxymethylcellulose (CMC) was autoclaved and was used as binding agent to effectively coat the sterilized seeds (4 ml CMC with 1 g seed). Surface sterilized seeds were immersed in bacterial suspension (1×10^8 CFU/mL) and CMC solution was added. Dipped seeds were kept overnight (10-12 h), air-dried for 3-4 h, sown in pots (10 in each), and were observed for germination and growth. Seeds without treatment were used as an experimental control. Plants were carefully uprooted after 60 days after sowing (DAS) and various plant growth parameters such as root length, shoot length, plant dry weight and fresh weight, spike length, number of grains per spike and the tiller numbers were analyzed.

3.11 Statistical analysis

All the data of plant growth parameters were statistically analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) at 5% level of significance ($\alpha = 0.05$) to compare the significant or insignificant difference between treatment means of plants (Gomez and Gomez 1984). Assessment of the statistical data was done with the help of MS Excel and software statistical package for the social sciences (SPSS) (2016) for windows.

RESULTS

4.1 Isolation of bacterial endophytes

In total 42 isolates were obtained from root tissues of *T. aestivum* collected from different sites (Fig. 11, 12 and Table 2). It was found that the isolated colonies showed assorted results in size, shape, color, and growth pattern on nutrient agar (NA) medium plates (Fig. 13 and Table 3).

4.2 Analysis of internal structure of wheat roots

Root tissues of collected wheat plants were analyzed for their internal structure by simple staining. Sections of root tissues were observed with various structural components such as inner and outer layer of cortex, symbiotic zone with various cells infected with PGPE and few cells without endophytes were also seen (Fig. 15). Similar structure was also observed under scanning electron microscopy (Fig. 16).

4.3 Morphological and physiological properties of isolated bacterial endophytes

Most of the bacterial endophytic isolates (73.8%) were found to be Gram negative in nature and 32 isolates showed positive results for motility (Fig. 14). Amongst them, the shape of only 2 isolates were cocci and remaining were rod shaped when observed through microscope. In SEM analysis, the size of isolates ranged from 1.50-1.75 μm in length and 0.50-0.58 μm in width (Fig. 17). Details of phenotypic characters of all the isolated bacterial endophytes is given in Table 3.

Most of the isolates showed blue coloration and produced alkali on nutrient agar plates supplemented with BTB while only few (9.5%) were found to be able to produce acid indicated by production of yellow color (Fig. 18). The physiological properties of endophytic isolates were checked by analyzing their growth on various growth conditions i.e., carbon sources, nitrogen sources, presence of salt stress, and different pH concentration. Most of the isolates (95.2%) were able to utilize applied carbon sources such as glucose, dextrose, lactose, galactose, sucrose and maltose (Table 4). 90% of the total isolates were also found to be positive for the utilization of nitrogen sources such as yeast extract, potassium nitrate, sodium nitrate, ammonium chloride, ammonium sulphate, and tryptophan (Table 5). It was found that all the bacterial isolates were able to grow upto 2% salt concentration whereas 95.2% isolates were able to form colonies in the presence of 4% NaCl. Among all the isolates 57.14% grew on plates containing 6% NaCl while 26.1% and 9.5% tolerated salt concentrations of 8% and 10% respectively. The isolates were also able to grow at pH 4-10 but only limited of them (59.52%) survived at pH 10.

The growth kinetics curve in the presence of salt stress for the selected endophytic isolates (AU15 and OC36) was drawn as both the isolates showed maximum PGP traits along with salt tolerance in the present study. Both the isolates were assessed for the growth at different NaCl concentrations and were found to be tolerant up to NaCl. The survival of the isolates was not affected upto 2% NaCl; however, salinity above 4% drastically reduced the growth rate as depicted in Fig. 22.

4.4 Biochemical characterization of endophytic isolates

Detection of indole (a by-product of tryptophan metabolism) relying upon the production of red dye rosindole under acidic conditions was assayed and it was found that only three isolates were positive for the indole production. All the endophytic isolates except one i.e., BN41 showed positive catalase activity by liberating oxygen on the addition of hydrogen peroxide. Enzymatic activities such as amylase, protease and lipase were investigated and showed by 54.76%, 30.95%, and 47.61% isolates respectively. Of the total 35.7 % isolates were found to be positive for utilization of citrate and displayed blue colored zone around the colonies. Study also revealed that all the 42 endophytic isolates were positive for ammonia production ability, and 13 isolates for MR test, 9 isolates for VP test were marked to be positive for the test. On the basis of all these biochemical tests, it was interpreted that the isolates were relatively diverse in nature and exhibited different bacterial putative endophytic communities (Fig. 18 and Table 7).

4.5 Plant growth promoting (PGP) characterization

4.5.1 Siderophore production

Production of high-affinity iron-chelating compounds known as siderophores was showed by 69% isolates and orange colored zone was observed around the bacterial colonies (Fig. 19). Quantitative analysis demonstrated highest siderophore production ability in PD25 with 42.2 psu and AU15 (39.9 psu) respectively as shown in Table 8.

4.5.2 IAA production

Ability of endophytic bacterial isolates to produce IAA was assayed on the basis of color change and spectrophotometric analysis. All the 42 endophytic isolates were checked for their potential to produce IAA and 22 (52.3%) isolates were able to utilize tryptophan and produce IAA. Isolate KA28 was recorded to produce maximum amount of IAA with value 122.9 µg/ml followed by OC36 (118.79 µg/ml) (Table 8).

4.5.3 Phosphate solubilization

The attribute of solubilizing insoluble inorganic phosphate compounds was shown by 45.2% of the isolates and among all the isolates AU15 showed maximum phosphate solubilisation ability (2.3 psi). Isolates OC37 showed the minimum clear zone with least solubilisation index of 0.7 psi.

4.5.4 Zinc solubilization

Among all the isolated endophytes, 30.95% were able to solubilize zinc and formation of clear zone around the colonies was spotted. Isolate OC36 exhibited maximum zinc solubilisation ability with 4.26 zsi followed by isolate BN39 with 3.86 zsi whereas minimum solubilisation was shown by isolate BN41 with 1.22 zsi.

4.5.5 HCN production

Isolates were tested for production of HCN and development of light brown to dark brown color was observed. HCN production by microbes has been postulated to play role in the biological control of phytopathogens and was displayed by none of the endophytic isolates.

4.5.6 Potassium solubilization

Among 42 isolates tested, only 8 (19%) have been found positive for K solubilization and were able to dissolve potassium from insoluble K-bearing minerals amended in the agar medium. Isolate PD25 caused maximum potassium solubilization in media supplemented with mica whereas OC37 showed minimum solubilization.

4.5.7 ACC deaminase assay

The endophytic isolates obtained from root tissues of wheat were checked for being able to grow on DF minimal salt medium supplemented with 3 mM ACC as a nitrogen source. Eight bacterial endophytic isolates i.e., MP2, NA6, AU15, CP18, PD22, KA28, KA31 and OC36 were found to be able to grow on the DF medium that indicated positive test result for ACC deaminase activity.

4.6 Screening of bacterial endophytes for nitrogen fixation

4.6.1 Growth on Jensen's medium

The ability of bacterial endophytic isolates to fix nitrogen was preliminary assayed on Jensen's medium and it was found that 25 isolates (59.5%) showed visible growth on the N free medium (Table 9).

4.6.2 PCR amplification of nifH

Twenty three of the forty-two isolates demonstrated to be positive for the *nifH* gene amplification yielded an end product of ~390 bp length as shown in Fig. 21; Table 9**.**

Whereas presence of strong visual bands of DNA was detected in seven bacterial isolates namely AU15, CP18, PD22, PD25, KA28, KA31, OC36.

4.6.3 Acetylene reduction assay

ARA method was used to study N_2 fixing abilities of the endophytic isolates and the rate of nitrogenase enzyme activity showed remarkable variation which ranged from 29.78 to 29.95 nmole C_2H_4 h^{-1} $culture^{-1}$. Maximum value of ethylene production was observed in the isolate PD25 and value obtained was 29.959 nmole C_2H_4 h^{-1} $culture^{-1}$ (Fig. 20). Isolate PD22 and KA28 showed 29.84 and 29.87 nmole C_2H_4 h^{-1} $culture^{-1}$ respectively whereas the lowest ARA activity was seen in the case of CP18 (29.78 nmole C_2H_4 h^{-1} $culture^{-1}$).

In total forty-two isolates were obtained in the study and were screened for their potential to fix N in the present study. On the basis of the results, seven endophytic isolates namely AU15, CP18, PD22, PD25, KA28, KA31, OC36 were selected for further characterization and experimental work.

4.7 Determination of endophytic nature of selected isolates

Colonization efficiency of selected isolates were investigated further and it was found that all the seven isolates were able to effectively reinfect the roots of host plant i.e., wheat and proliferated as endophyte which was confirmed after their re-isolation from root tissues of treated seedlings and scanning electron microscopy (Fig. 24 and Table 9). It was interpreted that the bacterial endophyte colonized the plants through cracks formed at the zone of root differentiation or emergence of lateral roots. No evidence of rhizospheric or endophytic colonization was seen in test control and this rule out the possibility of cross contamination.

4.8 Molecular identification by 16S rRNA gene sequencing and phylogenetic analysis

The seven endophytic isolates (AU15, CP18, PD22, PD25, KA28, KA31, OC36) were genotypically characterized and on the basis of phylogenetic analysis they were identified as strains from different genera. Phylogenetic tree was constructed by determining the closest evolutionary distances. The accession number were assigned to each of the isolates after submitting the gene sequences in the NCBI GenBank database (Table 12). 16S rRNA gene sequence analysis of the isolate AU15 showed 99% similarity with *Bacillus firmus* strain T20 (accession number MT457466.1) through BLASTn and the accession number of AU15 obtained from GenBank was OL872276 (Fig. 32). Endophytic isolate AU15 was confirmed as type strain by NAIMCC, India and assigned with the accession number of *B. firmus* NAIMCC-B-03040.

16S rRNA sequencing of OC36 showed 99% similarity with *Brevibacterium antiquum* strain AZGX-4 (Accession Number MF170850.1). Gene sequences were submitted to NCBI GenBank with accession number OL966902 and the isolate OC36 was subsequently also deposited to NAIMCC, India with the accession number *B. antiquum* NAIMCC-B-03041.

16S rRNA sequences of KA31 and CP18 showed 99% identity with *Alcaligenes faecalis* strain KMDH5 16S ribosomal RNA gene through NCBI-BLASTn (Fig. 26, 30). Molecular analysis of PD25, KA28, PD22 were also performed through 16S rRNA gene sequencing and the isolates were identified as *Proteus mirabilis, Pseudomonas marginalis, Priestia aryabhattai,* respectively (Fig. 27, 28, 29).

4.9 Pathogenicity test

Few isolates (CP18, PD25, and KA31) belonging to the genus of opportunistic human pathogens i.e., *Alcaligenes,* and *Proteus* were assayed for hemolytic activity prior to the pot culture experiments. It was observed that no zone of clearance was observed around the colonies after 10 days of incubation thereby suggesting that the strains were nonpathogenic and avirulent (Fig. 25).

The tested endophytic isolates did not exhibit enzymatic activities of protease and gelatinase as no zone of clearance was reported around the bacterial colonies which indicates that the strains were non-pathogenic and avirulent. The results of catalase tests showed positive reactions whereas lower effervescence also suggests the avirulency of endophytic isolates. In vivo laboratory experiments have proved the nonpathogenic nature of selected endophytic isolates whereas, the clinical trials will be done soon.

4.10 Application of isolates to assess their impact on the growth of wheat

Plant growth promoting activities of bacterial endophytes were evaluated for their ability to improve growth of wheat under saline conditions (EC 9.72 dS/m) and the seeds were inoculated with seven isolates (AU15, CP18, PD22, PD25, KA28, KA31, OC36) showing maximum PGP traits and salt tolerance abilities. The growth parameters of treated plants were statistically analyzed and exhibited significance difference in comparison to experimental control as shown in Table 14. The obtained results of pot experimental study clearly showed that all the endophytic isolates improved the plant growth parameters in respect to non-treated control under salt stressed conditions, however AU15 and OC36 were observed to be best plant growth promoters (Fig. 33). The seed bio priming with AU15 caused significant increase in plant growth comparatively to all other treatments and control plants; however, the treatment with OC36 also resulted in significantly higher growth in wheat plants. Germination rate of various treatments was analyzed and it was interpreted that AU15 displayed highest increase of 78.5% as compared to untreated control. Least effective result was obtained in plants treated with PD25. The data values of root length showed maximum increment of 156.1% in the AU15 inoculated plants which was 3.8% higher than plants treated with OC36. Two-fold increase in shoot length was reported in the plants treated with both the isolates AU15 and OC36 in comparison to control ones. Comparing the differences of fresh weight and dry weight with subjection to various treatments highest values of 84.5% and 163% were observed respectively in AU15 treated plants. Whereas OC36 enhanced crop productivity in a similar way by increasing plant’s fresh weight, and dry weight by 84%, and 162% comparing with untreated plants. Considering other treatments, all the treated plants showed increase in growth parameters while lowest effect was observed by PD25 inoculated plants with the increase in values of plant’s root length, shoot length, fresh weight, and dry weight by 102%, 25.20%, 55%, and 25.1% respectively, in comparison to control. Elucidating the effect of treatments on tiller numbers and spike length, significant differences were observed when endophytic isolates were inoculated to the plants. Wheat plants treated with AU15 showed 119.7% increase in tiller numbers while the isolate enhanced spike length from 4.25 cm (in control plants) to 8.03 cm. Tiller numbers and spike length were also increased by 99.4% and 74.8% respectively in the wheat plants treated with isolate OC36. The productivity of wheat is dependent on grain yield which directly correlates with the number of grains per spike. Considering the same, it was observed that grain number per spike significantly increased by 62.6% in AU15 inoculated plants while OC36 also enhanced the no. of grains by 52.3% in comparison to control.

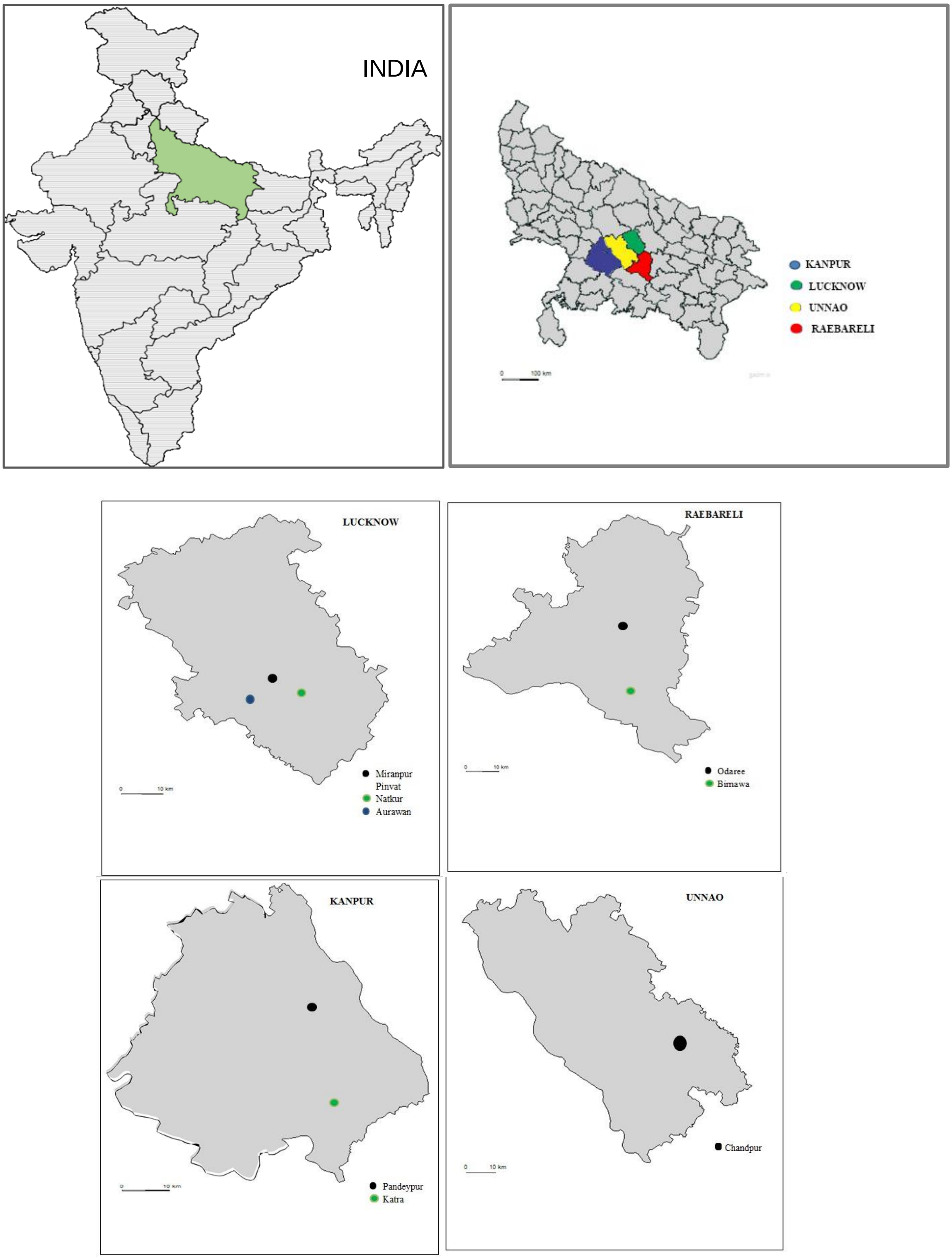


Fig. 11 Geographic locations of the study and sampling sites

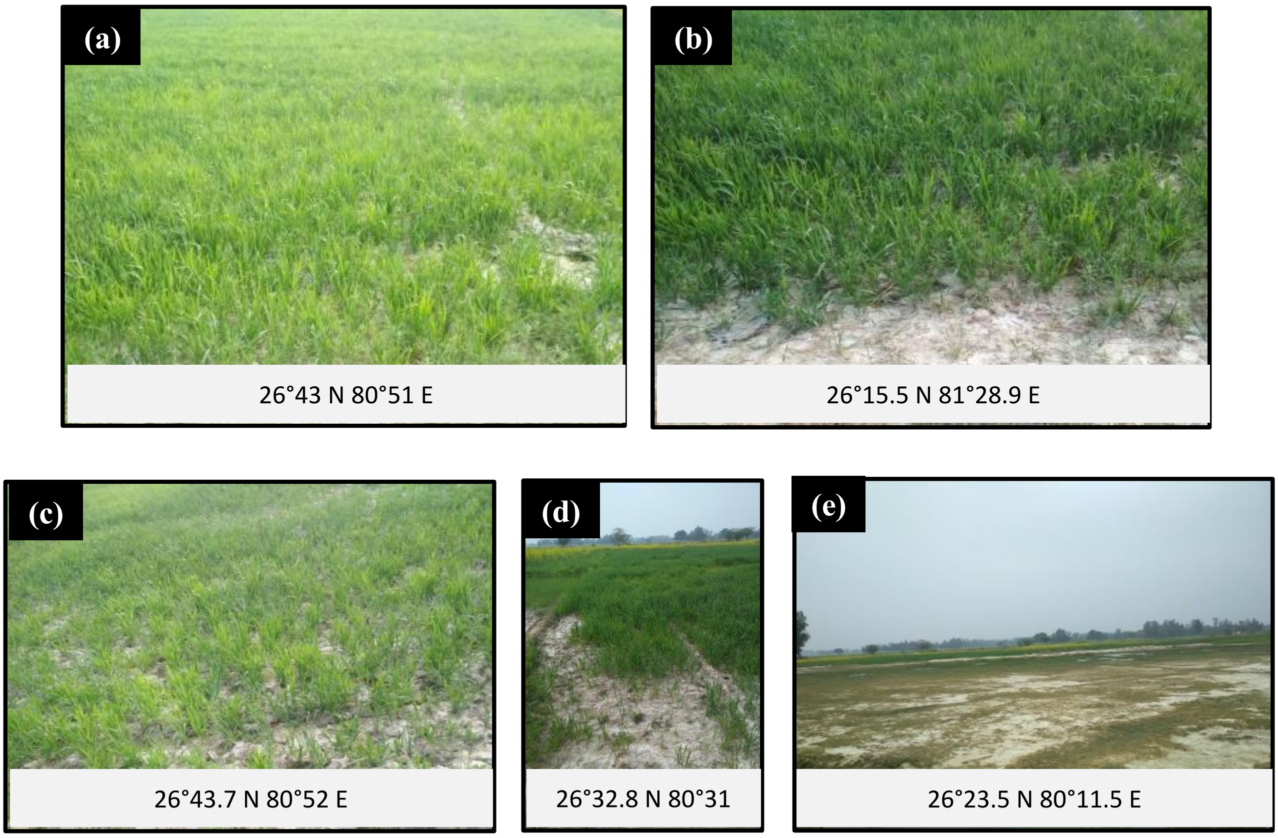


Fig. 12 Sampling sites of Uttar Pradesh
(a) Miranpur pinvat (b) Odaree (c) Natkur (d) Chandpur (e) Pandeypur

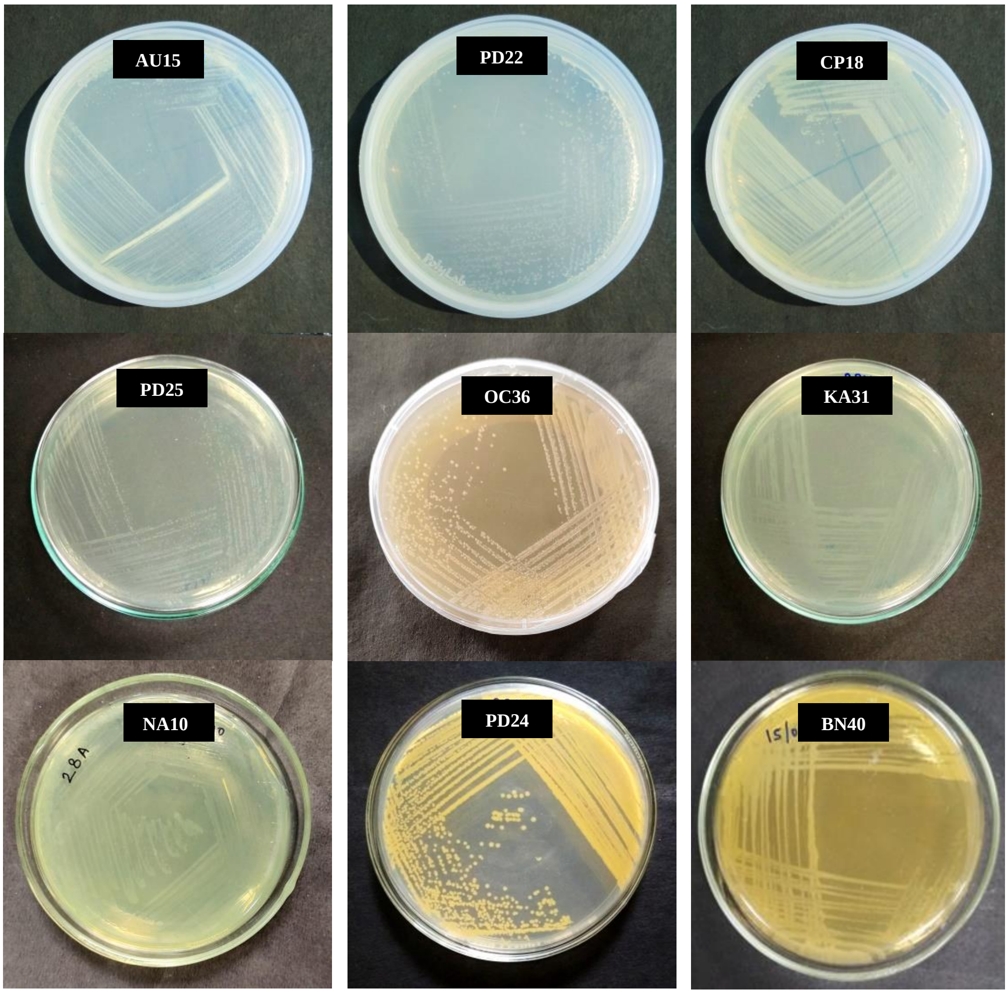
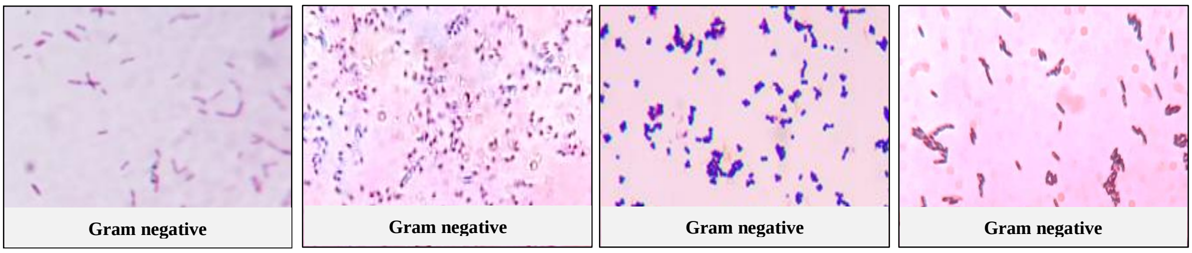


Fig. 13 Pure culture of bacterial endophytic isolates on nutrient agar medium



Gram negative

Gram negative

Gram negative

Gram negative

Fig. 14 Gram staining of bacterial endophytic isolates

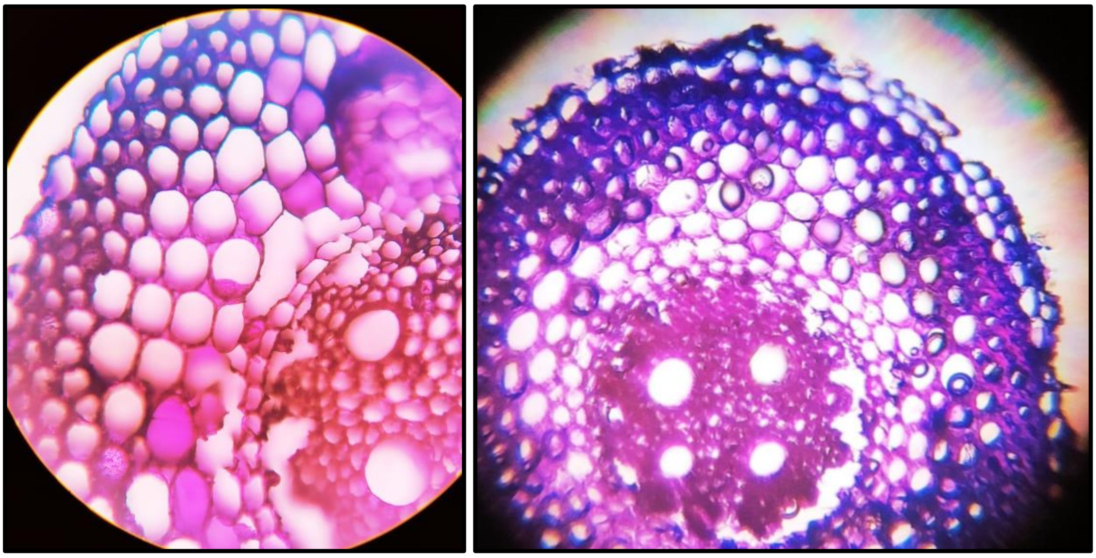


Fig. 15 Section of roots of wheat plant (simple staining)

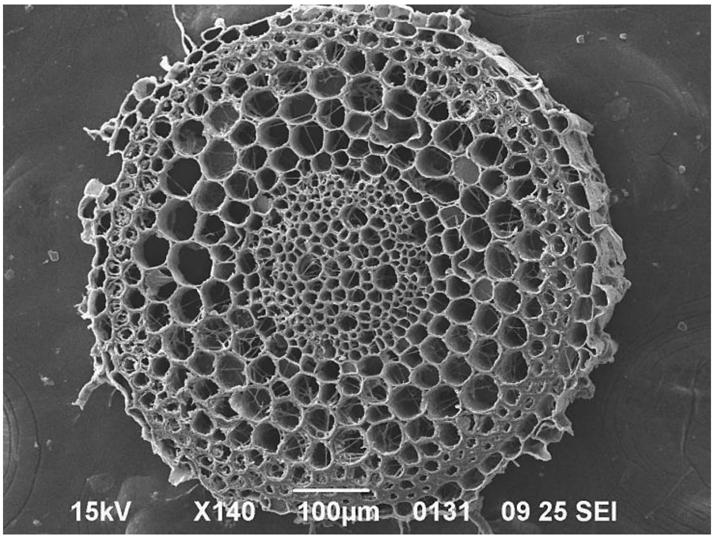


Fig. 16 Scanning electron micrographs showing internal structure of wheat roots

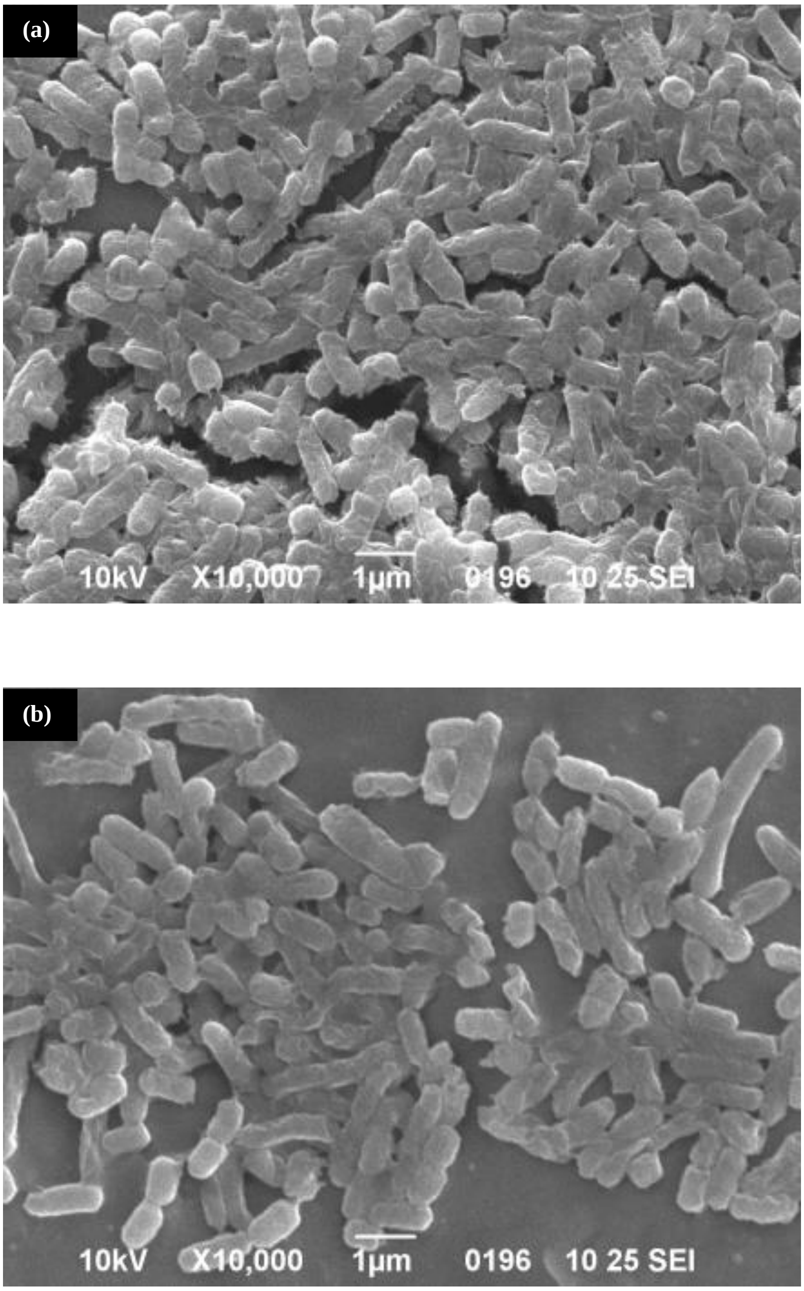
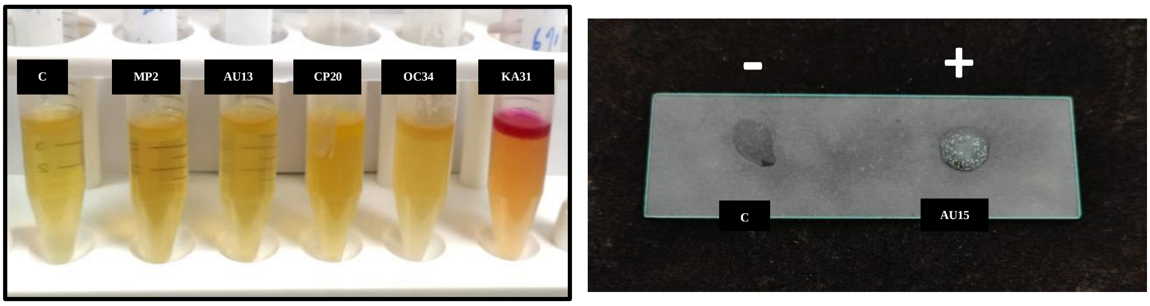
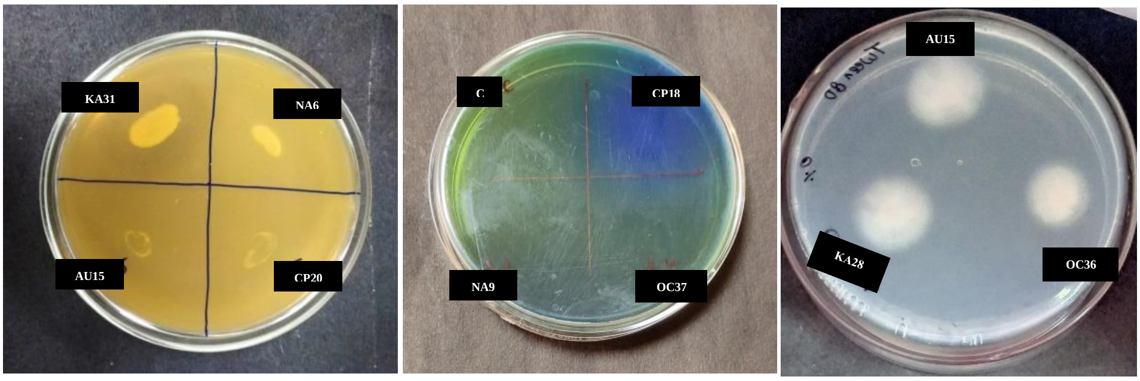


Fig. 17: Scanning electron micrograph of bacterial endophytic isolates (a) AU15 (b) OC36



Indole test

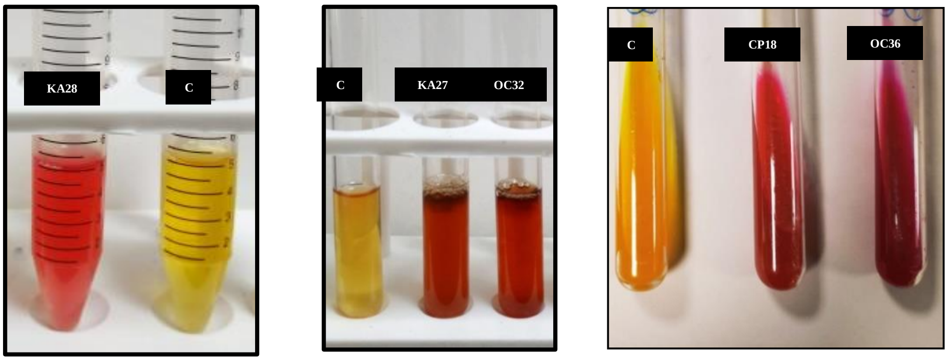
Catalase



Casein hydrolysis

Citrate utilization

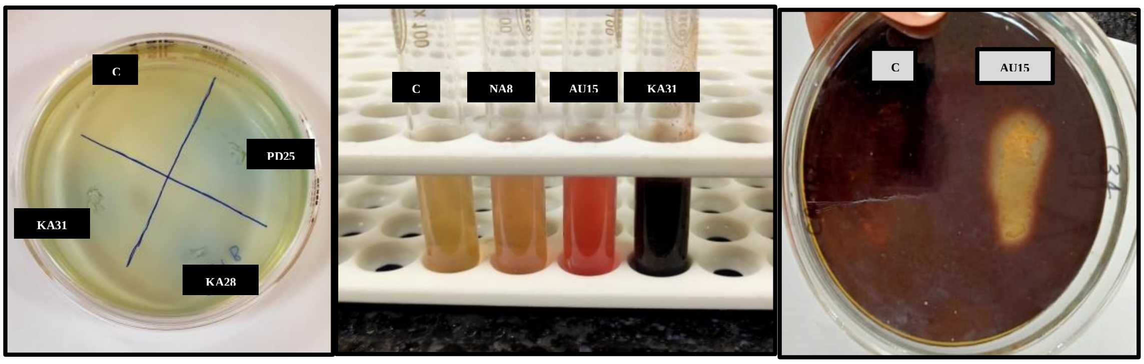
Lipase



Methyl red

Voges Proskauer

Urease

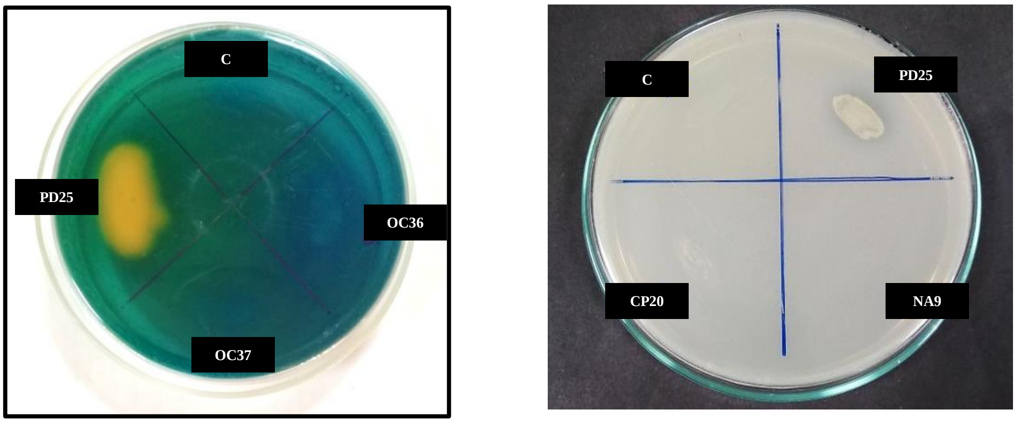


BTB test

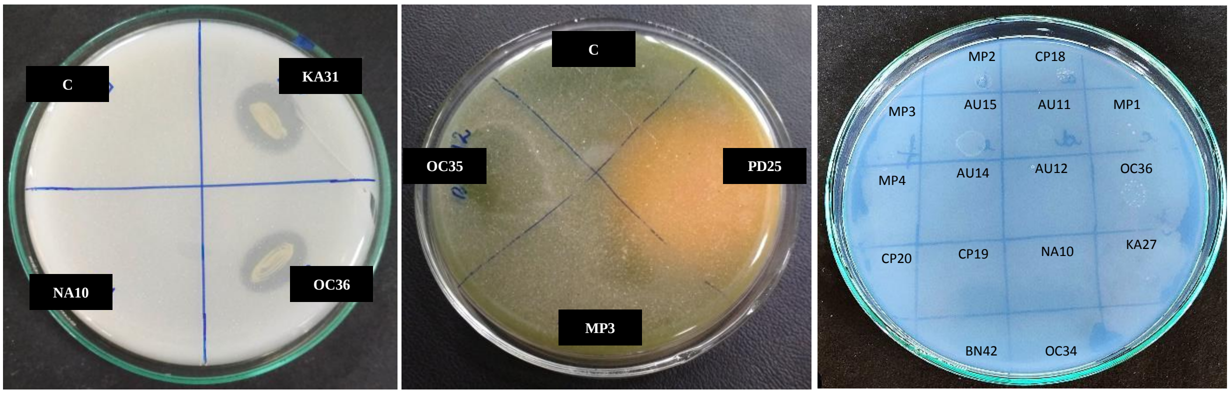
Nitrate reductase activity

Amylase

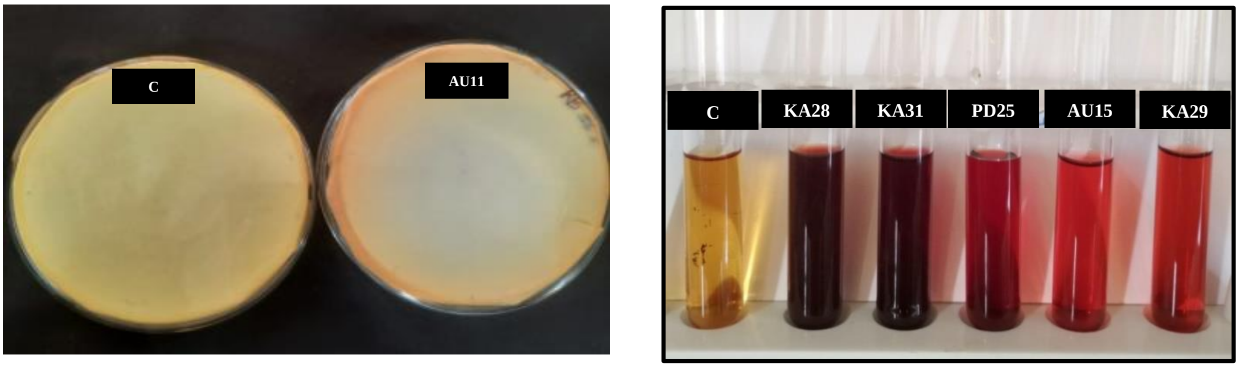
Fig. 18 Results of biochemical tests of isolates



Siderophore Production **Zinc solubilization**



Phosphate solubilization **K solubilization** **ACC deaminase assay**



HCN Production **IAA Production**

Fig. 19 Plant growth promoting characters of endophytic isolates

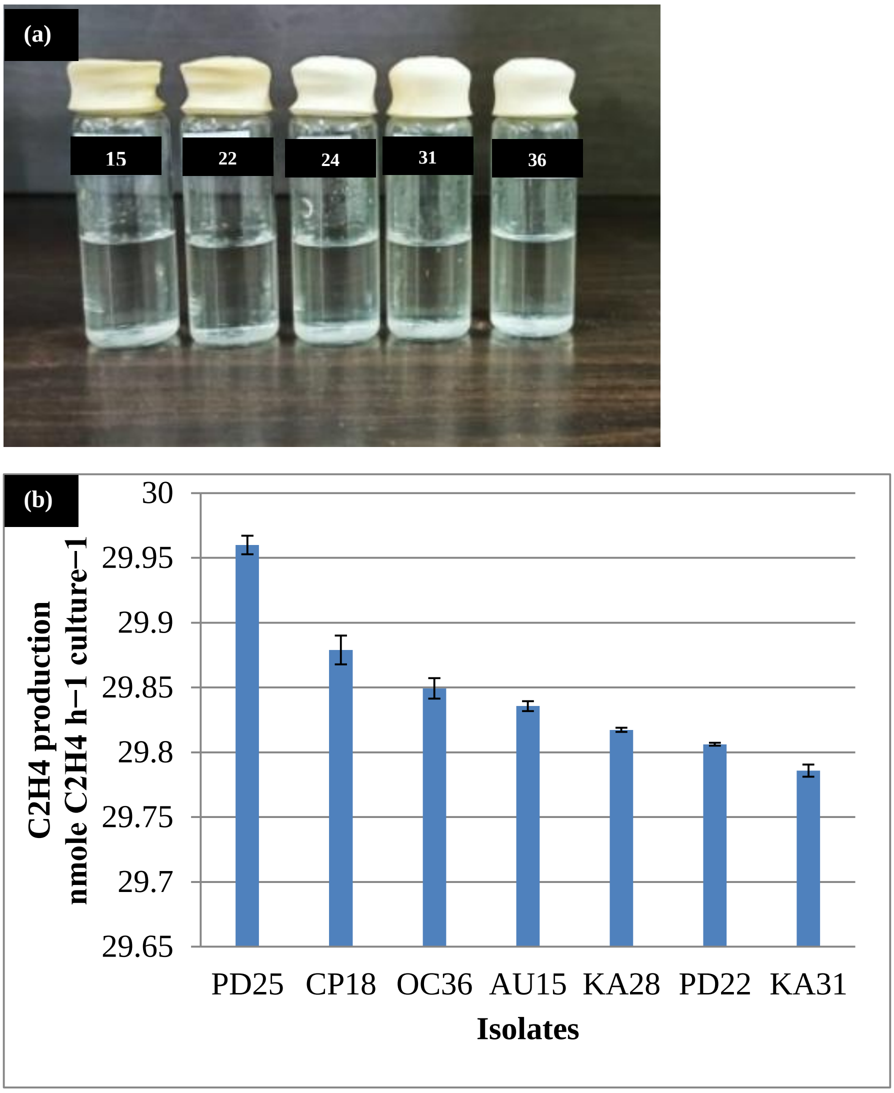


Fig. 20 Acetylene reduction assay (a) Culture tubes containing inoculated N free medium equipped with suba seal (b) Graphical representation showing production of ethylene by selected endophytic isolates

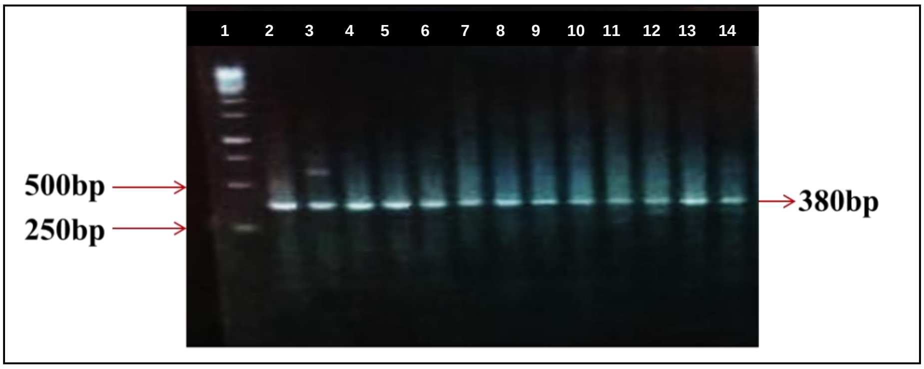


Fig. 21 Agarose gel electrophoresis analysis of PCR amplification products of *nifH* gene Numbers: 1 molecular marker; and 2 to 14 representing the isolates AU15, CP18, PD22, PD25, KA28, KA31, OC36, BN39, CP20, AU11, OC32, PD24, MP3

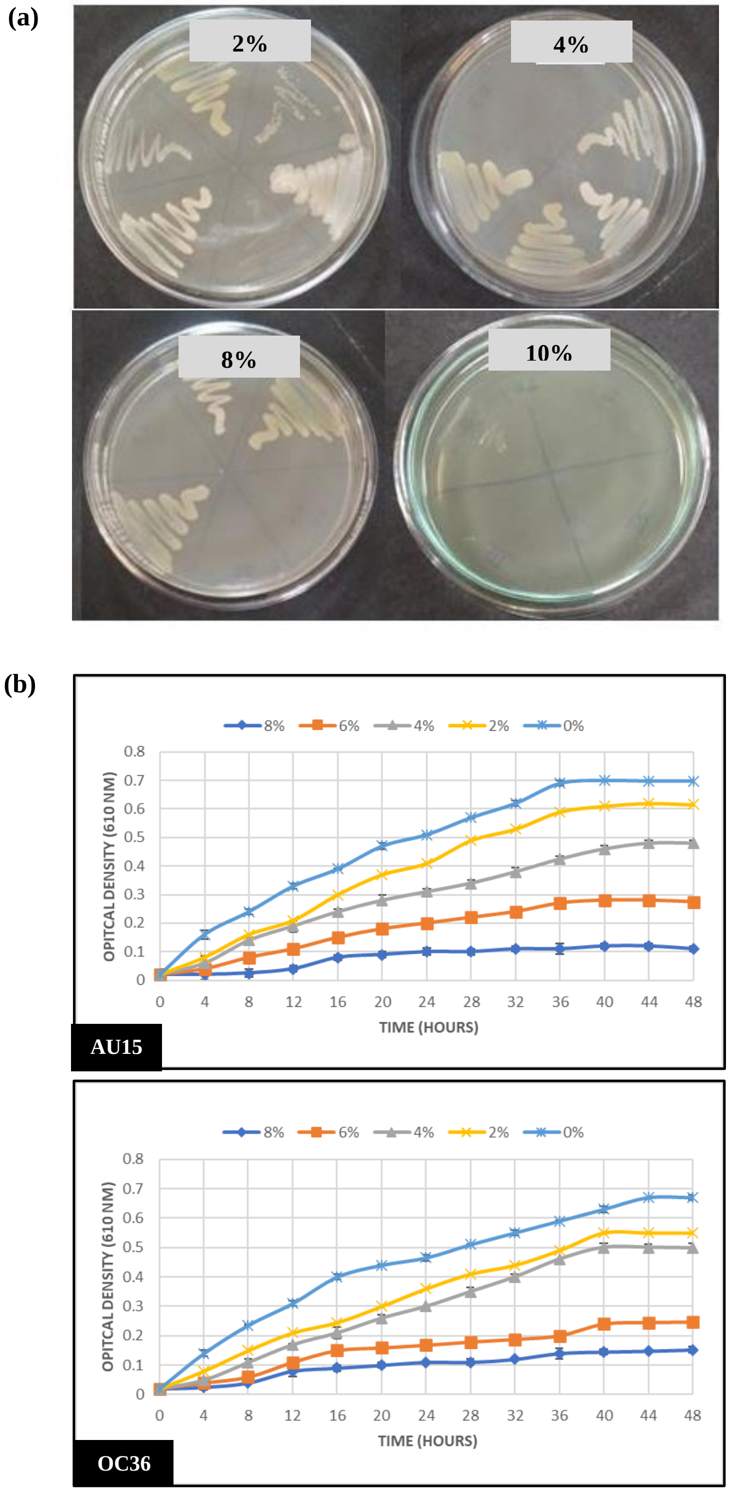


Fig. 22 Salt tolerance assay (a) Growth of bacterial endophytic isolates on agar medium plates containing 2%, 4%, 6% and 8% NaCl (b) Growth curve of AU15 and OC36 under different salt concentrations (2-8% NaCl) and non-saline control

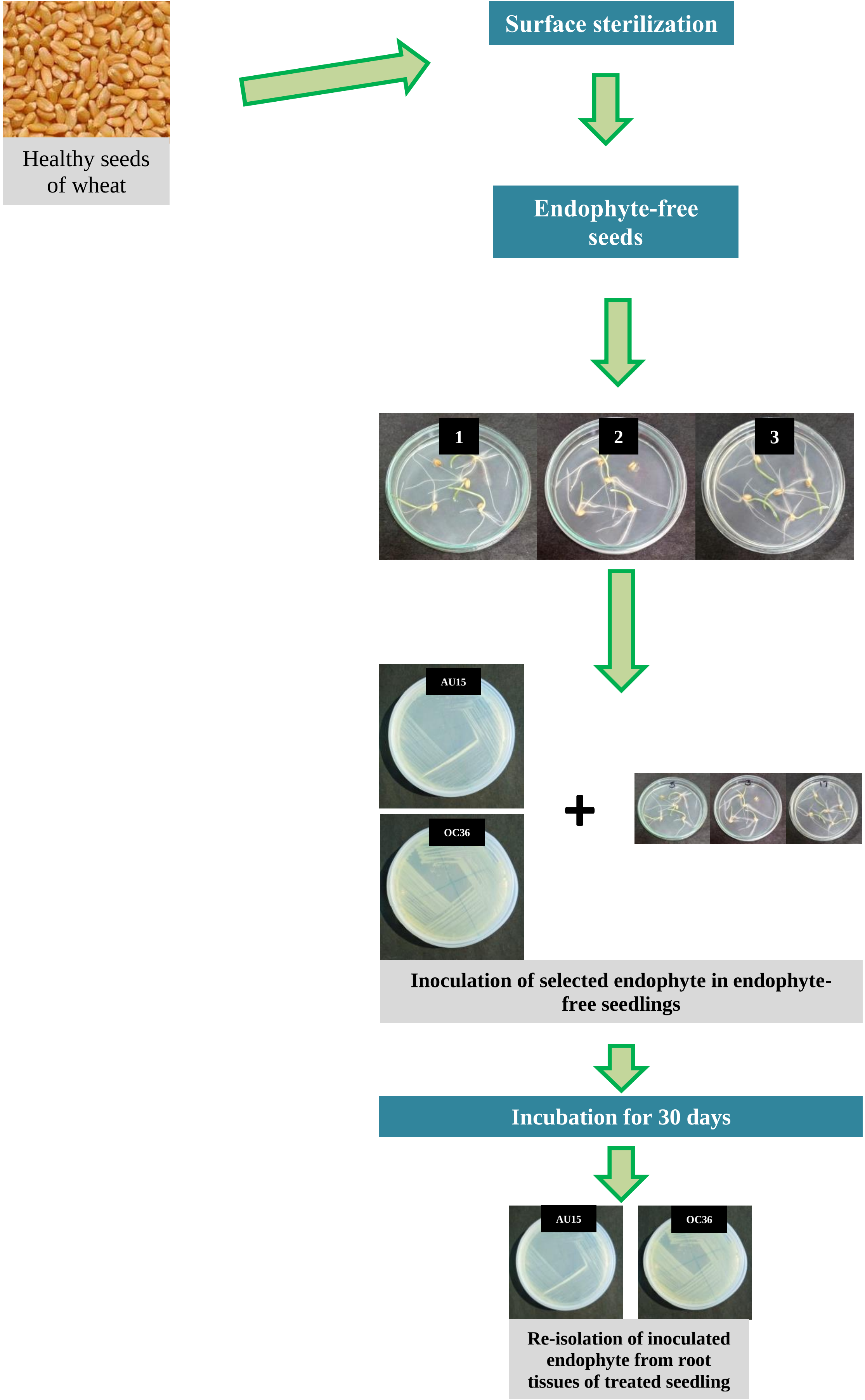


Fig. 23 Scheme of experiment done to determine the endophytic nature of isolates

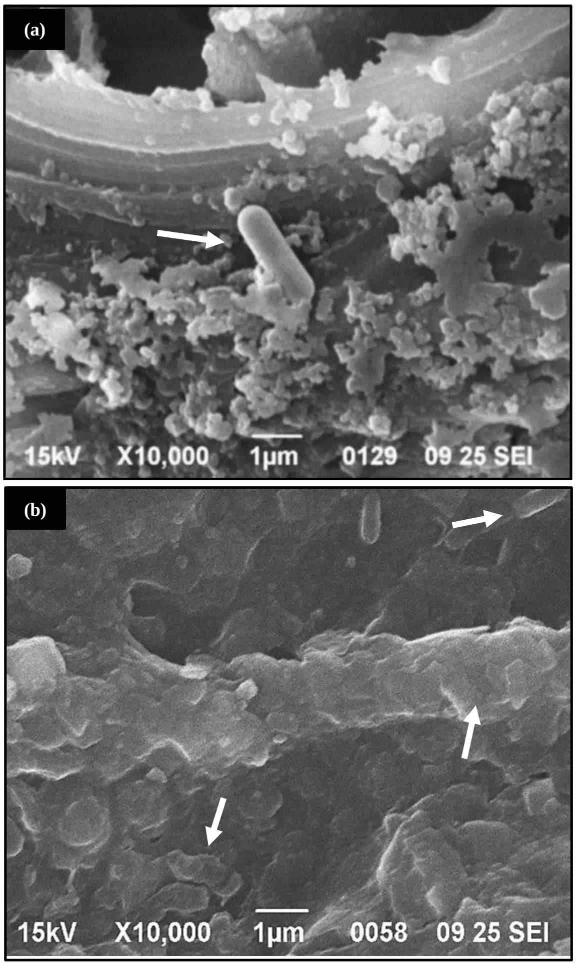


Fig 24: Scanning electron micrograph showing colonization of selected endophytic isolates in intercellular spaces of wheat roots (as indicated by arrow)
(a) Isolate AU15 (b) Isolate OC36

Fig 25: Pathogenicity test showing non-hemolytic activity of isolates on blood agar

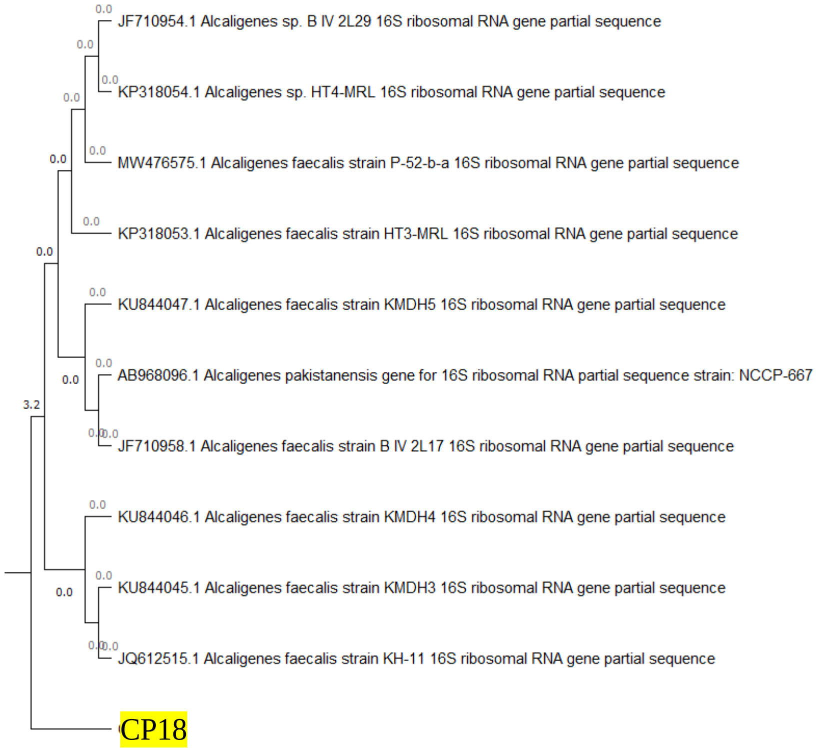


Fig 26: Phylogenetic tree of isolate CP18

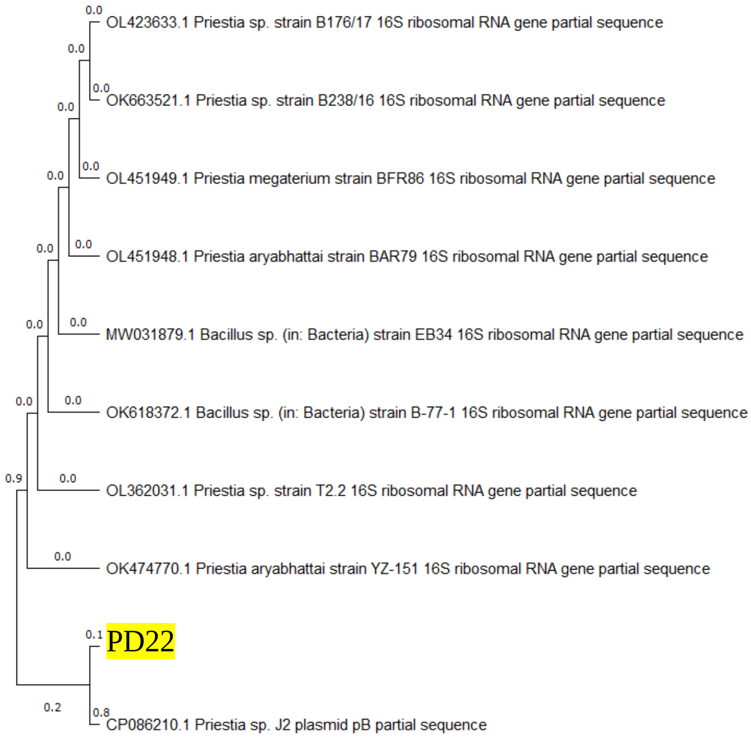


Fig. 27 Phylogenetic tree of isolate PD22

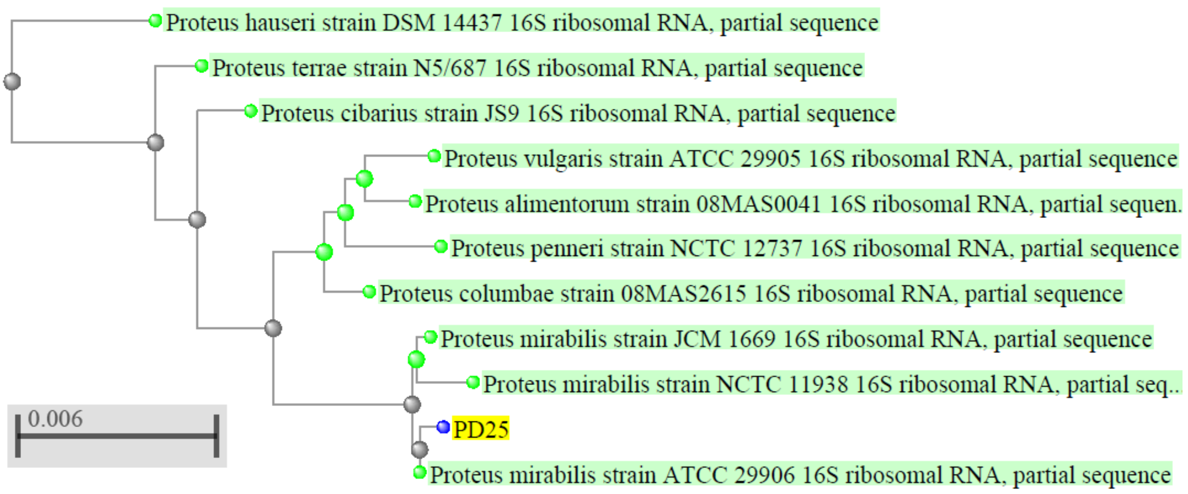


Fig. 28 Phylogenetic tree of isolate PD25

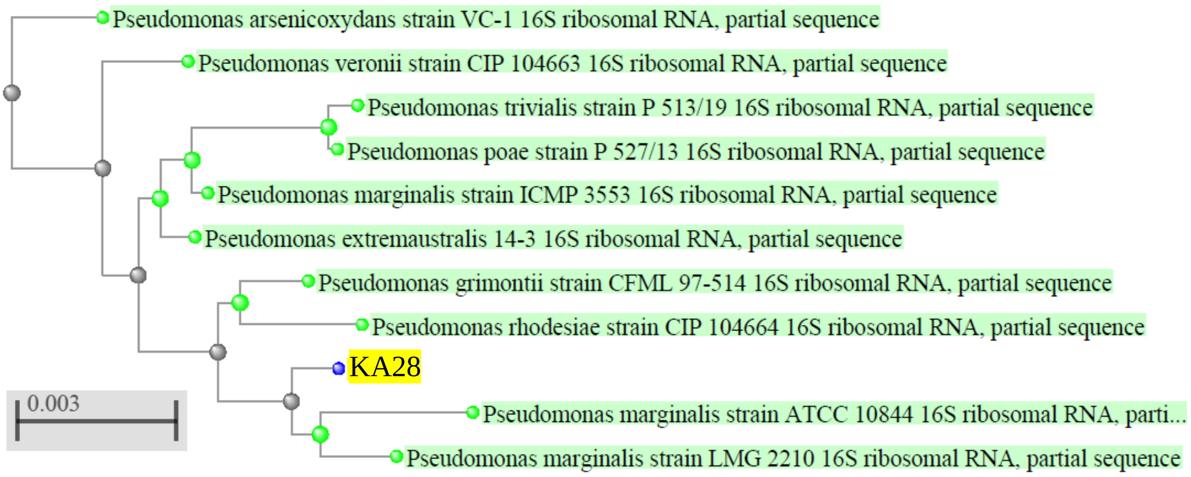


Fig. 29 Phylogenetic tree of isolate KA28

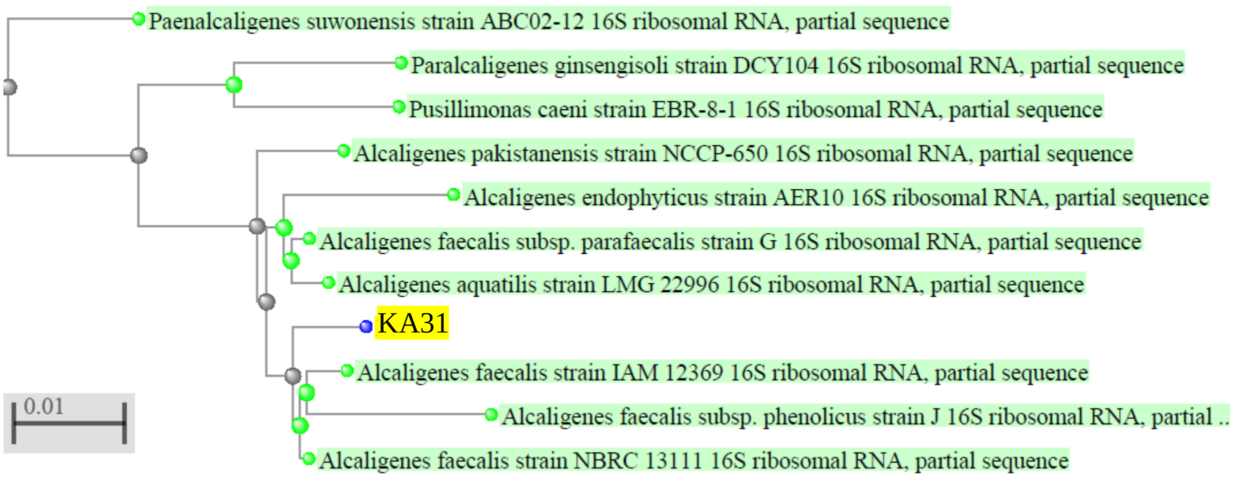


Fig. 30 Phylogenetic tree of isolate KA31

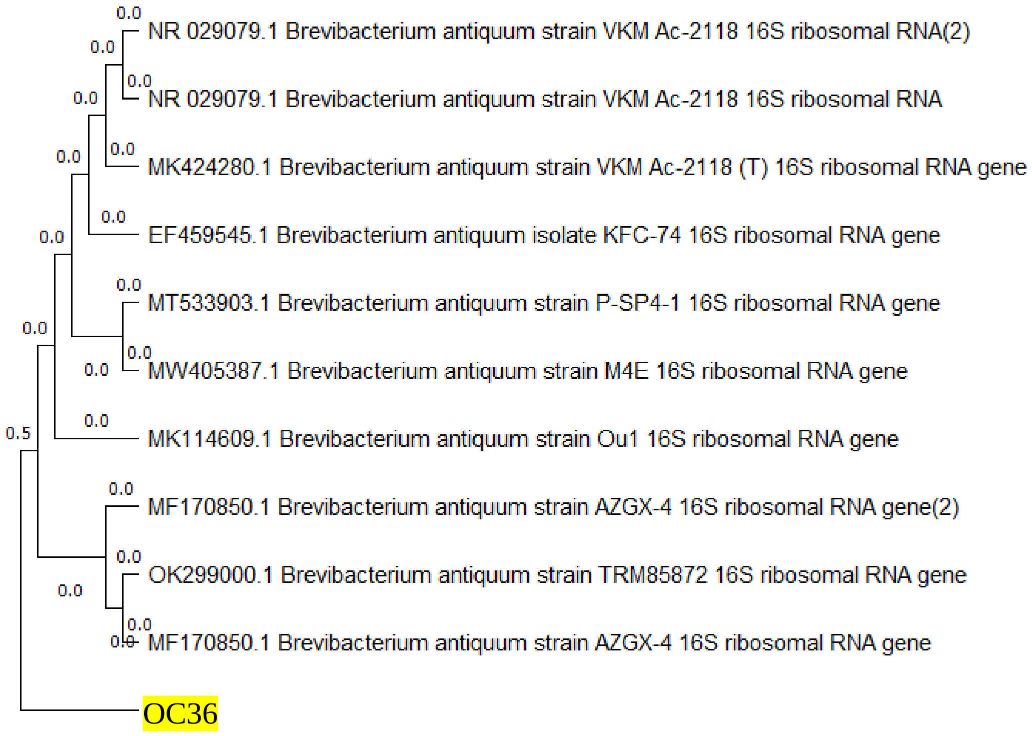


Fig. 31 Phylogenetic tree of isolate OC36

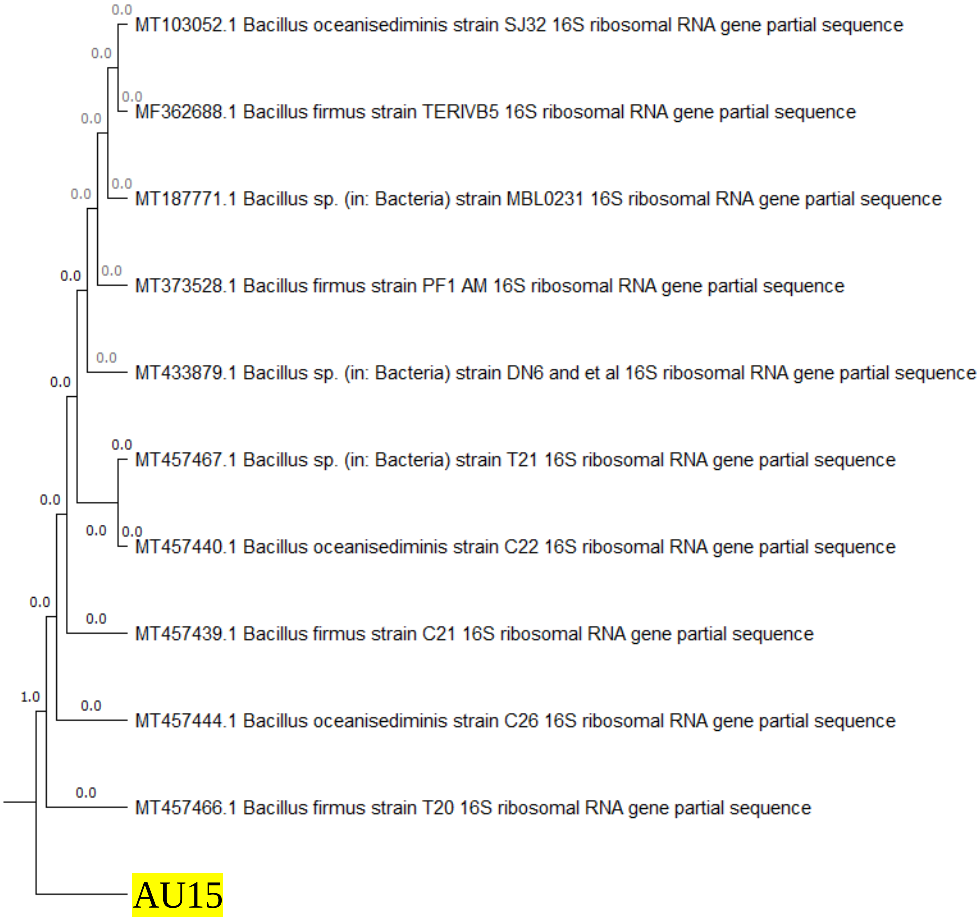


Fig. 32 Phylogenetic tree of isolate AU15

Fig. 33 Application of isolates on wheat under saline conditions (a) Plant phenotypes in pots after treatment with endophytic isolates (b) Root and shoot growth of wheat plants after 45 days of sowing

Table. 2 Details of sampling sites

S. No.	Collection sites		Geographical location	Number of isolates	Name of isolates
	District	Village			
1.	Lucknow	Miranpur Pinvat	26°43 N 80°51 E	5	MP1, MP2, MP3, MP4, MP5
		Natkur	26°43.7 N 80°52 E	5	NA6, NA7, NA8, NA9, NA10
		Aurawan	26°42.65 N 80°49.2 E	5	AU11, AU12, AU13, AU14, AU15
3.	Unnao	Chandpur	26°32.8 N 80°31 E	5	CP16, CP17, CP18, CP19, CP20
2.	Kanpur	Pandeypur	26°23.5 N 80°11.5 E	6	PD21, PP22, PP23, PP24, PP25, PP26
		Katra	26°11.26N 80°10.05 E	5	KA27, KA28, KA29, KA30, KA31
4.	Raebareli	Odaree	26°15.5 N 81°28.9 E	6	OC32, OC33, OC34, OC35, OC36, OC37
		Biranawan	26°10.5 N 81°28.2 E	5	BN38, BN39, BN40, BN41, BN42

Table. 3 Phenotypic characteristics of the endophytic isolates

S.No.	Isolates	Growth rate	Motility	Microscopic observation	
				Gram Nature	Shape
1.	MP1	Fast	+	positive	rod
2.	MP2	Slow	+	negative	rod
3.	MP3	Slow	-	negative	rod
4.	MP4	Fast	+	negative	rod
5.	MP5	Fast	+	negative	rod
6.	NA6	Fast	+	negative	rod
7.	NA7	Fast	+	negative	rod
8.	NA8	Slow	+	negative	rod
9.	NA9	Fast	-	negative	rod
10.	NA10	Slow	+	positive	rod
11.	AU11	Slow	+	positive	rod
12.	AU12	Slow	+	positive	rod
13.	AU13	Slow	+	negative	rod
14.	AU14	Fast	-	negative	rod
15.	AU15	Fast	+	positive	rod
16.	CP16	Slow	-	positive	rod
17.	CP17	Fast	+	negative	rod
18.	CP18	Fast	+	negative	rod
19.	CP19	Fast	-	negative	rod
20.	CP20	Fast	+	negative	cocci
21.	PD21	Slow	+	negative	rod
22.	PD22	Fast	+	positive	rod
23.	PD23	Fast	-	negative	rod
24.	PD24	Fast	-	negative	rod
25.	PD25	Fast	+	negative	rod
26.	PD26	Slow	+	negative	rod
27.	KA27	Fast	+	negative	rod
28.	KA28	Fast	+	negative	rod
29.	KA29	Slow	+	negative	rod
30.	KA30	Slow	+	negative	rod
31.	KA31	Fast	+	negative	rod
32.	OC32	Slow	+	negative	rod
33.	OC33	Slow	-	negative	rod
34.	OC34	Slow	+	negative	rod
35.	OC35	Slow	+	negative	rod
36.	OC36	Fast	+	positive	rod
37.	OC37	Fast	+	negative	rod
38.	BN38	Fast	+	negative	rod
39.	BN39	Fast	+	positive	rod
40.	BN40	Fast	-	positive	cocci
41.	BN41	Slow	+	negative	rod
42.	BN42	Fast	-	positive	rod

+ = positive for test, - = negative for test

Table. 4 Carbon source utilization pattern of the endophytic isolates

S. No.	Isolates	Carbon sources					
		Glucose	Dextrose	Lactose	Galactose	Sucrose	Maltose
1.	MP1	+	+	+	+	+	+
2.	MP2	+	+	+	+	+	+
3.	MP3	+	+	+	+	+	+
4.	MP4	+	+	+	+	+	+
5.	MP5	+	+	+	+	+	+
6.	NA6	+	+	+	+	+	+
7.	NA7	+	-	+	+	+	-
8.	NA8	+	+	+	+	+	+
9.	NA9	+	+	+	+	+	+
10.	NA10	+	+	+	+	+	+
11.	AU11	+	+	+	+	+	+
12.	AU12	+	+	+	+	+	+
13.	AU13	+	+	+	+	+	+
14.	AU14	+	+	+	+	+	+
15.	AU15	+	+	+	+	+	+
16.	CP16	+	+	+	+	+	+
17.	CP17	+	+	+	+	+	+
18.	CP18	+	+	+	+	+	+
19.	CP19	+	+	+	+	+	+
20.	CP20	+	+	+	+	+	+
21.	PD21	+	+	+	+	+	+
22.	PD22	+	+	+	+	+	+
23.	PD23	+	+	+	+	+	+
24.	PD24	+	+	+	+	+	+
25.	PD25	+	+	+	+	+	+
26.	PD26	+	+	+	+	+	-
27.	KA27	+	+	+	+	+	+
28.	KA28	+	+	+	+	+	+
29.	KA29	+	+	+	+	+	+
30.	KA30	+	+	+	+	+	+
31.	KA31	+	+	+	+	+	+
32.	OC32	+	+	+	+	+	+
33.	OC33	+	+	+	+	+	+
34.	OC34	+	+	+	+	+	+
35.	OC35	+	+	+	+	+	+
36.	OC36	+	+	+	+	+	+
37.	OC37	+	+	+	+	+	+
38.	BN38	+	+	+	+	+	+
39.	BN39	+	+	+	+	+	+
40.	BN40	+	+	+	+	+	+
41.	BN41	+	+	+	+	+	+
42.	BN42	+	+	+	+	+	+

+ =utilized, - = not utilized

Table. 5 Nitrogen source utilization pattern of the endophytic isolates

S. No.	Isolates	Nitrogen sources					
		Yeast extract	NH_4Cl	$NaNO_3$	NH_4So_4	KNO_3	Tryptophan
1.	MP1	+	+	+	+	+	+
2.	MP2	+	+	+	+	+	+
3.	MP3	+	+	-	+	+	+
4.	MP4	+	+	+	+	+	+
5.	MP5	+	+	+	+	+	+
6.	NA6	+	+	+	+	+	+
7.	NA7	+	+	+	+	+	+
8.	NA8	+	+	+	+	+	+
9.	NA9	+	+	+	+	+	+
10.	NA10	+	+	+	+	+	+
11.	AU11	+	+	+	+	+	+
12.	AU12	+	+	+	+	+	+
13.	AU13	+	+	+	+	-	+
14.	AU14	+	+	+	+	+	+
15.	AU15	+	+	+	+	+	+
16.	CP16	+	+	+	+	+	+
17.	CP17	+	+	+	+	+	+
18.	CP18	+	+	+	+	+	+
19.	CP19	+	+	+	+	+	+
20.	CP20	+	+	+	+	+	+
21.	PD21	+	+	+	+	+	+
22.	PD22	+	+	+	+	+	+
23.	PD23	+	+	+	+	+	+
24.	PD24	+	+	+	+	-	+
25.	PD25	+	+	+	+	+	+
26.	PD26	+	+	+	+	+	+
27.	KA27	+	+	+	+	+	+
28.	KA28	+	+	+	+	+	+
29.	KA29	+	+	+	+	+	+
30.	KA30	+	+	+	+	+	+
31.	KA31	+	+	+	+	+	+
32.	OC32	+	+	+	+	+	+
33.	OC33	+	+	+	+	-	+
34.	OC34	+	+	+	+	+	+
35.	OC35	+	+	+	+	+	+
36.	OC36	+	+	+	+	+	+
37.	OC37	+	+	+	+	+	+
38.	BN38	+	+	+	+	+	+
39.	BN39	+	+	-	+	+	+
40.	BN40	+	+	+	+	+	+
41.	BN41	+	+	+	+	+	+
42.	BN42	+	+	+	+	+	+

+ =utilized, - = not utilized

Table. 6 Growth pattern of isolates under different salt concentrations and pH

S.No.	Isolates	Salt concentration (%)					pH variants			
		2	4	6	8	10	4	6	8	10
1.	MP1	+	+	+	-	-	-	+	+	+
2.	MP2	+	+	+	+	+	-	+	+	+
3.	MP3	+	+	+	-	-	+	+	+	-
4.	MP4	+	-	-	-	-	-	+	+	+
5.	MP5	+	+	+	+	+	-	+	+	-
6.	NA6	+	+	+	+	-	-	+	+	-
7.	NA7	+	+	-	-	-	+	+	+	-
8.	NA8	+	+	+	-	-	-	+	+	+
9.	NA9	+	+	+	-	-	-	+	+	-
10.	NA10	+	+	+	-	-	-	+	+	+
11.	AU11	+	+	+	-	-	-	+	+	-
12.	AU12	+	+	+	+	-	-	+	+	+
13.	AU13	+	+	-	-	-	+	-	+	+
14.	AU14	+	+	+	+	-	-	+	+	+
15.	AU15	+	+	+	+	-	-	+	+	-
16.	CP16	+	+	-	-	-	-	+	+	-
17.	CP17	+	+	+	+	+	-	+	+	+
18.	CP18	+	+	-	-	-	+	+	+	+
19.	CP19	+	+	-	-	-	-	+	+	+
20.	CP20	+	+	+	+	-	-	+	+	-
21.	PD21	+	+	+	-	-	+	+	+	+
22.	PD22	+	+	+	+	-	+	-	+	+
23.	PD23	+	+	+	-	-	+	+	+	-
24.	PD24	+	+	-	-	-	-	+	+	-
25.	PD25	+	+	-	-	-	-	+	+	+
26.	PD26	+	+	-	-	-	-	+	+	+
27.	KA27	+	+	+	-	-	-	-	+	+
28.	KA28	+	+	+	-	-	+	+	+	+
29.	KA29	+	+	-	-	-	+	+	+	+
30.	KA30	+	+	-	-	-	-	+	+	+
31.	KA31	+	+	+	-	-	+	+	+	+
32.	OC32	+	+	-	-	-	-	+	+	+
33.	OC33	+	+	+	-	-	-	+	+	-
34.	OC34	+	+	-	-	-	+	+	+	-
35.	OC35	+	+	+	-	-	-	+	+	-
36.	OC36	+	+	+	+	-	-	+	+	+
37.	OC37	+	+	-	-	-	-	+	+	-
38.	BN38	+	+	+	+	-	-	-	+	+
39.	BN39	+	+	-	-	-	+	+	+	-
40.	BN40	+	+	-	-	-	+	+	+	+
41.	BN41	+	-	-	-	-	-	+	+	+
42.	BN42	+	+	+	+	+	-	+	+	-

+ = positive for test, - = negative for test

Table. 7 Biochemical characters of isolates

S. No.	**Isolates**	**Biochemical test**											
		Indole production	**Catalase**	**Protease**	**Citrate utilization**	**Methyl red**	**Voges Proskauer**	**Urease**	**Nitrate reductase activity**	**Amylase**	**Lipase**	**Gelatinase**	**Ammonia production**
1.	**MP1**	-	+	++	+	-	-	-	+	-	+	-	++
2.	**MP2**	-	+	-	+	++	++	+	-	+	+	-	++
3.	**MP3**	-	+	-	-	-	-	+	+	-	+	-	++
4.	**MP4**	+	+	-	-	-	-	+	-	+	-	+	+
5.	**MP5**	-	+	-	+	+	-	-	-	+	-	+	+
6.	**NA6**	-	+	+	-	-	+	-	-	-	+	-	+
7.	**NA7**	-	+	-	-	+	-	-	-	+	+	-	+
8.	**NA8**	-	+	+	-	-	-	+	+	+	-	-	+
9.	**NA9**	-	+	-	+	-	-	+	+	+	-	-	+
10.	**NA10**	-	+	-	-	-	-	-	+	-	+	-	+
11.	**AU11**	-	+	-	-	++	-	+	+	-	-	-	+
12.	**AU12**	-	+	-	-	-	+	-	+	-	+	+	+
13.	**AU13**	-	+	-	+	-	++	-	-	+	-	+	+
14.	**AU14**	-	+	-	-	+	-	-	-	-	-	+	+
15.	**AU15**	-	+	+	-	-	-	-	+	+	+	+	+
16.	**CP16**	-	+	+	+	-	-	+	+	-	+	+	+
17.	**CP17**	-	+	-	-	-	++	+	+	-	+	+	+
18.	**CP18**	-	+	-	+	-	-	+	-	+	-	-	+
19.	**CP19**	-	+	-	-	-	-	-	-	-	-	-	+
20.	**CP20**	-	+	+	-	++	-	-	-	+	+	-	+

21.	**PD21**	-	+	-	-	++	-	+	-	-	-	-	+
22.	**PD22**	-	+	-	-	+	-	-	-	+	+	-	+
23.	**PD23**	-	+	-	+	+	-	+	-	-	-	+	+
24.	**PD24**	-	+	+	+	-	-	+	+	-	-	+	+
25.	**PD25**	-	+	++	+	+	-	+	+	+	-	+	+
26.	**PD26**	-	+	+	-	-	+	-	-	-	-	+	+
27.	**KA27**	-	+	+	-	-	+	-	+	+	+	-	+
28.	**KA28**	-	+	+	-	+	-	-	+	+	+	-	+
29.	**KA29**	-	+	-	-	-	-	-	+	-	-	+	+
30.	**KA30**	-	+	-	+	-	-	+	+	-	-	+	+
31.	**KA31**	+	+	++	+	-	-	-	+	+	-	-	++
32.	**OC32**	-	+	-	-	-	+	+	-	-	+	+	+
33.	**OC33**	-	+	-	-	-	-	+	-	+	+	-	+
34.	**OC34**	-	+	-	-	-	-	-	+	-	+	-	+
35.	**OC35**	-	+	-	+	-	-	+	-	+	-	+	+
36.	**OC36**	-	+	-	-	-	-	+	-	-	+	+	+
37.	**OC37**	-	+	-	-	+	-	-	-	+	-	-	+
38.	**BN38**	-	+	+	+	-	-	-	-	-	-	-	+
39.	**BN39**	+	+	-	-	-	+	+	-	+	+	-	+
40.	**BN40**	-	+	-	-	-	-	+	+	-	+	-	+
41.	**BN41**	-	-	-	+	-	-	+	+	+	-	+	+
42.	**BN42**	-	+	-	-	+	-	-	+	+	-	-	+

- = negative for test , + = positive for test, ++ = strongly positive for test

Table. 8 Evaluation of plant growth promoting attributes of bacterial isolates (qualitative and quantitative)

S. No.	Isolates	PGP characters						
		K Solubilization (ksi)	**Zinc solubilization (zsi)**	**Phosphate solubilization (psi)**	**IAA production (µg/ml)**	**Siderophore Production (psu)**	**HCN production**	**ACC deaminase**
1.	**MP1**	-	-	-	+ (85.77±0.04)	++ (30.38±0.07)	-	-
2.	**MP2**	-	-	+ (1.5±0.92)	+ (82.91±0.20)	+ (17.97±0.02)	-	+
3.	**MP3**	+ (3.14±0.18)	-	+ (1.31±0.70)	+ (70.12±0.12)	++ (33.43±0.13)	-	-
4.	**MP4**	-	-	-	+ (75.68±0.27)	+ (27.81±0.06)	-	-
5.	**MP5**	-	-	+ (1.1±0.48)	-	+ (25.13±0.18)	-	-
6.	**NA6**	-	-	-	-	+ (21.12±0.22)	-	+
7.	**NA7**	-	-	-	-	+ (18.95±0.86)	-	-
8.	**NA8**	-	+ (1.49±0.23)	+ (0.8±0.29)	++ (98.15±0.61)	+ (25.13±0.38)	-	-
9.	**NA9**	-	-	-	+ (55.71±0.14)	+ (29.21±0.74)	-	-
10.	**NA10**	-	-	+ (1.03±0.11)	+ (56.63±0.08)	+ (23.17±0.42)	-	-
11.	**AU11**	+ (5.72±0.67)	++ (2.96±0.08)	-	-	-	-	-
12.	**AU12**	-	-	-	+ (54.6±0.22)	+ (21.33±1.07)	-	-
13.	**AU13**	-	-	-	++ (70.1±0.06)	+ (28.15±0.09)	-	-
14.	**AU14**	-	++ (3.04±0.12)	+ (1.2±0.43)	-	+ (12.67±0.17)	-	-
15.	**AU15**	-	++ (3.1±0.15)	++ (2.3±0.38)	++ (102.54±0.17)	+++ (39.9±0.15)	-	+
16.	**CP16**	-	-	+ (1.5±0.74)	+ (69.12±0.06)	-	-	-
17.	**CP17**	++ (7.21±0.05)	-	-	+ (82.14±0.13)	+ (23.12±0.11)	-	-
18.	**CP18**	-	-	+ (1.8±0.67)	-	++ (30.4±0.08)	-	+
19.	**CP19**	-	-	-	-	++ (34.26±0.74)	-	-
20.	**CP20**	-	++ (3.3±0.07)	-	-	-	-	-

21.	**PD21**	-	-	+ (0.9±0.27)	-	-	-	-
22.	**PD22**	+ (5.79±0.41)	-	-	-	+ (28.3±0.06)	-	+
23.	**PD23**	-	-	-	-	+ (15.35±0.03)	-	-
24.	**PD24**	+ (6.01±0.07)	-	+ (1.5±1.2)	-	+ (10.4±0.18)	-	-
25.	**PD25**	+++ (11.03±0.13)	+ (2.98±0.06)	-	+ (75.47±0.21)	+++ (42.28±0.29)	-	-
26.	**PD26**	-	-	-	-	-	-	-
27.	**KA27**	-	++ (3.15±0.20)	-	++ (99.66±0.11)	+ (21.47±1.45)	-	-
28.	**KA28**	-	+ (2.72±0.33)	-	+++ (122.93±0.13)	+ (28.33±0.17)	-	+
29.	**KA29**	-	-	-	-	+ (13.62±0.22)	-	-
30.	**KA30**	-	-	+ (1.8±0.29)	-	+ (17.41±0.47)	-	-
31.	**KA31**	-	-	+ (1.96±0.86)	++ (115.52±0.05)	++ (36.33±0.25)	-	+
32.	**OC32**	-	+ (1.99±0.08)	-	-	-	-	-
33.	**OC33**	+ (10.46±0.91)	-	-	-	+ (22.35±0.17)	-	-
34.	**OC34**	-	-	-	-	-	-	-
35.	**OC35**	-	+ (1.48±0.19)	-	-	-	-	-
36.	**OC36**	-	+++ (4.26±0.19)	+ (1.4±0.12)	+++ (118.79±0.61)	+ (20.15±0.08)	-	+
37.	**OC37**	+ (3.10±0.12)	-	+ (0.7±0.08)	++ (105.14±0.01)	-	-	-
38.	**BN38**	-	-	+ (1.8±0.03)	+ (65.47±0.13)	-	-	-
39.	**BN39**	-	+ (3.86±0.12)	-	-	-	-	-
40.	**BN40**	-	-	+ (1.93±0.15)	+ (54.25±0.63)	+ (25.37±0.12)	-	-
41.	**BN41**	-	+ (1.22±0.3)	-	+ (76.77±0.02)	-	-	-
42.	**BN42**	-	-	-	-	-	-	-

- = negative for test , + = positive for test, ++ = strongly positive for test, psi= phosphate solubilisation index, zsi= zinc solubilisation index, psu= percent siderophore unit

Table. 9 Screening of diazotrophs amongst isolated bacterial endophytes

S.No.	Isolates	Growth on Jensen's medium	PCR amplification of *nifH* gene
1.	MP1	-	-
2.	MP2	-	-
3.	MP3	+	+
4.	MP4	+	+
5.	MP5	-	-
6.	NA6	-	-
7.	NA7	+	+
8.	NA8	-	-
9.	NA9	+	+
10.	NA10	+	+
11.	AU11	+	+
12.	AU12	-	-
13.	AU13	-	-
14.	AU14	+	-
15.	AU15	++	+
16.	CP16	+	-
17.	CP17	+	-
18.	CP18	++	+
19.	CP19	-	-
20.	CP20	+	+
21.	PD21	-	-
22.	PD22	++	+
23.	PD23	+	+
24.	PD24	-	+
25.	PD25	++	+
26.	PD26	-	-
27.	KA27	-	-
28.	KA28	++	+
29.	KA29	-	-
30.	KA30	-	-
31.	KA31	++	+
32.	OC32	+	+
33.	OC33	+	+
34.	OC34	-	-
35.	OC35	-	-
36.	OC36	++	+
37.	OC37	+	+
38.	BN38	-	+
39.	BN39	+	+
40.	BN40	+	-
41.	BN41	+	+
42.	BN42	+	+

++ = strongly positive, + = positive, - = negative

Table. 10 Results of ARA of selected diazotrophic endophytes

S.No.	Isolates	Nitrogenase activity (nmole C2H4 h^{-1} $culture^{-1}$)
1.	AU15	29.79294
2.	CP18	29.78583
3.	PD22	29.84931
4.	PD25	29.95989
5.	KA28	29.8789
6.	KA31	29.80614
7.	OC36	29.8173

Table. 11 Re-infection assay of selected endophytic isolates on their host plant i.e., wheat

S.No.	Isolates	Re infection test
1.	**AU15**	+
2.	**PD22**	+
3.	**PD23**	+
4.	**PD24**	+
5.	**PD25**	+
6.	**KA28**	+
7.	**KA31**	+

- = negative for colonization, + = positive for colonization

Table. 12 NCBI-Genbank submission and culture collection accession number details of isolates

S. No.	Isolates	Organism	Name of nearest Homology	% Identity	Accession number (GenBank)	Culture collection accession number (NAIMCC, Mau, India)
1.	**AU15**	***Cytobacillus firmus***	***Bacillus firmus* strain T20 16S ribosomal RNA gene**	**99.85%**	**OL872276**	**NAIMCC-B-03040**
2.	**OC36**	***Brevibacterium antiquum***	***Brevibacterium antiquum* strain AZGX-4 16S ribosomal RNA gene**	**99.85%**	**OL966902**	**NAIMCC-B-03041**
3.	PD25	*Proteus mirabilis*	*Proteus mirabilis* strain ATCC 29906 16S ribosomal RNA	99.93%	MW405826	-
4.	KA28	*Pseudomonas marginalis*	*Pseudomonas marginalis* strain LMG 2210 16S ribosomal RNA	99.64%	OL662916	-
5.	KA31	*Alcaligenes faecalis*	*Alcaligenes faecalis* strain KMDH5 16S ribosomal RNA gene	99.92%	OL423363	-
6.	CP18	*Alcaligenes faecalis*	*Alcaligenes faecalis* strain KMDH5 16S ribosomal RNA gene	99.92%	OL966931	-
7.	PD22	*Priestia aryabhattai*	*Bacillus aryabhattai* strain ZJJH-2 16S ribosomal RNA gene	100.00%	OL872259	-

Table. 13 Physico-chemical properties of soil used for pot study

Parameter	Value
pH	8.0
Electrical Conductivity (ds/m)	9.72
Organic carbon content (g/kg)	4.1
Nitrogen (g/kg)	0.13
Phosphorous (mg/kg)	42.1
Potassium (mg/kg)	155
Microbial biomass (mg/kg)	101.9
Organic matter (g/kg)	5.3

Table. 14 *In-vitro* study of selected isolates on agronomic growth parameters of wheat

Treatments	Root length (cm)	Shoot length (cm)	Fresh weight (g/plant)	Dry weight (g/plant)	Tiller numbers	Spike length (cm)	No. of grains per spike	Germination (%)
T1	4.42±0.26^{a}	20.73±0.87^{a}	11.97±0.17^{a}	1.59±0.14^{a}	1.67±0.57^{a}	4.25±0.52^{a}	22.33±0.57^{a}	46.66±5.77^{a}
T2	8.95±0.32^{b}	25.92±0.39^{b}	18.55±0.78^{b}	1.99±0.95^{a}	2.33±1.52^{a}	5.10±0.99^{ab}	32.33±1.24^{b}	53.33±5.77^{ab}
T3	9.07±0.15^{b}	32.32±0.35^{c}	19.64±0.89^{bc}	3.38±0.45^{b}	2.67±0.57^{a}	5.86±0.29^{b}	32.33±2.51^{b}	60±0.00^{bc}
T4	10.17±0.25^{c}	38.06±0.49^{d}	20.29±0.28^{cd}	3.66±0.08^{b}	2.67±1.52^{a}	6.91±0.28^{c}	32.67±1.52^{bc}	63.33±5.77^{cd}
T5	10.24±0.21^{c}	38.24±0.22d^{e}	21.32±0.29^{de}	3.99±0.58^{bc}	3 ±1.00^{a}	7.20±0.62^{c}	33.33±2.08^{bc}	70±0.00^{de}
T6	**11.32±0.54^{e}**	**40.03±0.25^{f}**	**22.09±0.11^{e}**	**4.19±0.24^{c}**	**3.67±0.57^{a}**	**8.03±0.25^{c}**	**36.33±3.05^{c}**	**83.33±5.77^{f}**
T7	10.57±0.37^{cd}	39.10±0.85^{ef}	21.50±1.31^{de}	4.13±0.54^{bc}	3.33±1.15^{a}	7.23±0.68^{c}	33.83±1.79^{bc}	73.33±5.77^{e}
T8	10.90±0.45^{de}	39.43±0.30^{f}	22.03±0.92^{e}	4.17±0.61^{bc}	3.33±0.57^{a}	7.43± 0.75^{c}	34.03±1.96^{bc}	76.66±5.77^{ef}

Data are means of ten replicates with standard error. Same letters denote statistically insignificant difference while treatments with different letters have significant difference in the values ($P < 0.05$). T1- Untreated control, T2- PD25, T3- KA31, T4- KA28, T5-CP18, T6- AU15, T7- PD22, T8- OC36.
**data means of three blocks*
Values in bold indicate the most effective treatment

DISCUSSION

Land degradation is a global challenge that affects people and ecosystems decreasing the long-term ability of soils to provide humans with services, including future food production and is affected by both climate change and contributions to it (Kopittke et al., 2019). The continuous increasing trend of soil salinity along with uncurbed soil erosion by wind and water demands an economical, sustainable, and large-scale remediation strategy to amend the lands in a limited time. Microorganisms are well known to mitigate the effects of salinity and this microbe-mediated stress tolerance in plants has been an eco-friendly approach for better crop yield (Arora and Vanza 2017, Mishra et al., 2017b). The diversity and beneficial characteristics of endophytic microbial communities along with their intimate mutualistic association with host plant have been studied extensively by researchers (de Araújo et al., 2021; Elmagzob et al., 2019; Nair and Padmavathy 2014; Fernandes et al, 2015). Plant endophytes are not only involved as key players in framing the structure and fertility of wastelands including saline or sodic soils but also play a significant role in defining the health status of plants (Etesami and Beattie, 2017). Reports available on plant growth-promoting abilities of endophytes provide substantial evidence for their application as bio inoculants in the cultivation of crops (Horrigan et al., 2002; Santoyo et al., 2016; ALKahtani et al., 2020). However, their application in agricultural systems and the commercial production in the amelioration of salinity stress is still in the initial stage and has not been much elucidated yet.

Soil salinization is a major process of land degradation that has threatened the productivity of soil as well as reduced the quality of several agricultural crops.

Vaishnav et al., (2016) states that plants are the first in the food chain to get affected by abiotic soil stresses, and this results in an overall effect on the food availability. Wheat is one of the important staple crops among the three significant cereals which ensure food security at the global level. The grain is in high demand in countries undergoing urbanization and industrialization (Shewry and Hey, 2015). Cultivation of wheat is highly persistent in terms of resources used and associated energy input worldwide. Wheat plants have high agricultural management flexibility and can be planted in a wide range of agro-climatic conditions (Jan et al., 2010). Intensive use of land including rigorous agricultural farming may result in degradation of soil; hence restoring natural conditions or ecosystems by reclamation and priorly improving the soil properties with the use of biological fertilizers is highly needed. In the field, where the salinity escalates to 100 mM NaCl, rice (*Oryza sativa*) will die before maturity, whereas wheat will produce lessened productivity and reduced yield (Salt Tolerance Database reproduced on USDA-ARS, 2005). Despite this, further improvements in salt tolerance of cereal crops are undoubtedly needed to sustain the food production as soil salinity and sodicity severely constrain the production of wheat crop in many regions of the world (Genc et al., 2019; Munns et al., 2006). The present study deals with the identification and characterization (phenotypic, biochemical, and molecular) of nitrogen fixing bacterial endophytes isolated from root tissues of collected wheat plants. Salt-tolerant bacterial endophytes were isolated from roots of wheat (*T. aestivum*) and were screened for their nitrogen fixing abilities and PGP attributes. The selected multi-PGP endophytic bacterial isolates were also investigated for their efficacy in improving biomass parameters of inoculated host plants i.e., wheat by conducting in vivo pot experiments under saline conditions.

The potential endophytic isolates i.e., AU15 and OC36 which have been selected on the basis of PGP properties and salt-tolerance levels were subjected to halotolerance assay to determine the effect of salt on bacterial growth. It was found that no effect on the growth pattern was observed for both the isolates upto 2% NaCl; however, growth rate significantly reduced in the presence of 8% NaCl. Isolates that are able to survive in the presence of high salt concentrations (up to 8% NaCl) are designated as moderately halotolerant bacterium as also discussed in earlier reports (Ventosa et al. 1998; Sharma et al., 2016); therefore, AU15 and OC36 can be considered as moderately halotolerant endophytic isolates. Afridi et al., (2019) isolated salt tolerant endophytes namely *Kocuria rhizophila* and *Cronobacter sakazakii* and both the bacterial isolates showed tolerance against different levels of salinity stress from 5%-15% NaCl. Another plant growth-promoting endophytic bacterium *Microbacterium* sp. tolerated to up to 4–6% salt concentration and was isolated from cultivable seed endophytes of rice (Walitang et al., 2017).

Semi-arid regions of Lucknow and adjoining districts were selected as the sampling site in the present study. In total, 42 morphologically distinct colonies of bacteria associated with the root tissues of *T. aestivum* were obtained as putative endophytic isolates. Morphological features of the endophytic isolates widely varied and most of them produced round and circular shaped colonies with raised margins. 31 bacterial isolates (73.8%) were Gram negative, while 11 (26.2%) appeared to be Gram positive under the light microscope. The phenotypic characterization showed that the majority of isolates (61.90%) were fast growers with 3 hrs mean generation time, while the remaining isolates were found to be slow growers having mean generation time of 8.6 hrs. Other studies also supported this categorization and reported that colonies with a generation time of <4 hrs are considered to be the fast growers while the isolates having generation time (>4 hrs) are called slow growers (Leslie and Summerell, 2008; Jida and Asefa, 2012). The optimum pH level for the growth of endophytic isolates was observed to be 7 but some (59.52%) of them were also able to flourish on pH 9 and pH 10. Characterization and identification of microbes cannot rely on a single characteristic and should be based upon overall phenotypic and biochemical characterization patterns in order to determine the taxonomical details of the isolates (Pitt and Barer 2012). Therefore, in the present investigation, the physiological and biochemical properties of isolates were also determined under various cultural conditions that included tests such as utilization of various carbon and nitrogen sources, growth at pH variants, salt concentrations, enzymatic tests, etc. All the endophytic isolates showed diverse results in the utilization of a variety of organic compounds as carbon sources such as glucose, dextrose, lactose, galactose, sucrose, and maltose. Consumption of carbon sources was studied using Biolog plates in another study and the results indicated that endophytic bacteria were strongly adaptable in obtaining both carbon and nitrogen from varying environments (Liu et al., 2020). The current study accounted that the isolates were able to utilize a diverse range of carbon sources while the most preferred carbon source was glucose as reported earlier as well (Zinniel et al., 2002; Xie et al., 2021). Osono and Takeda, (2001) also noted the ability of endophytic microorganisms to utilize various organic compounds encompassing carbon sources which enable endophytes to degrade structural components of plants such as wood and plant leaf litter. The utilization pattern of nitrogen sources by plant endophytes has also been checked by researchers (Bajwa and Read, 1986; Mahmoud and Narisawa, 2013; Kumar et al., 2016) and in the present study, all the endophytic isolates were also able to exploit most of the applied nitrogen sources comprising of yeast extract, tryptophan, KNO_3, and NH_4Cl. In the same context, Botelho and Mendonça-Hagler (2006) further added that the utilization of diverse sugars along with amino acids helps the microorganisms to consume root exudates and establish themselves in the rhizosphere aiding colonization within the plants. N promoted the growth of plants in a concentration-dependent manner, with minor differences being detected among the different nitrogen sources as reported by Naher et al., (2018). In the present work, most of the endophytic isolates exhibited tolerance to salinity and 40 isolates (95.2%) were found to be able to grow up to 4% salt concentration, whereas 57.14% isolates displayed colonial growth up to 6% salinity level in growth media. Salinity tolerance serves as one of the characterization tools for plant growth promoting microorganisms including endophytes and potential application of salt-tolerant endophytes in the management of stresses in crop plants has been primarily explored in the present study (Verma et al., 2021; Afridi et al., 2021; Ali et al., 2014). The endophytic bacterial isolates were characterized as per the standard protocols of Bergey’s Manual of Systematic Bacteriology (Garrity 2005) and the production of extracellular enzymes such as catalase, lipase, amylase, citrate, protease and cellulase were also assayed. These enzymes function to obtain nutrients, elicit defense mechanisms against pathogens, and could also establish a unique niche for ecological adaptation during symbiosis with the host plant (Khan et al., 2016; Uzma et al., 2016).

Plant growth promoting endophytes are widely known for maintaining and boosting plant health and play important roles in plant-microbial interactions and plant-growth regulation (Santoyo et al., 2016; Cueva-Yesquén et al., 2021). Endophytes are regarded to have an advantage over rhizospheric microorganisms since living within a plant's tissues gives them an opportunity to always be in¨contacẗ with the complex plant’s tissue cells and thereby exerting a direct beneficial effect on the plant growth and development (Marquez-Santacruz et al., 2010). Similar to other plant growth-promoting microorganisms, endophytes also play a significant role in facilitating the plant growth in agriculture, horticulture and silviculture as well as in environmental cleanup i.e., phytoremediation (Backer et al., 2018; Eid et al., 2021). They share a unique symbiotic relationship with their host plant and stimulate plant growth either directly or indirectly. The direct mechanisms involved in growth promotion include acquisition of nutrients (nitrogen, phosphorous, and iron) from the environment and regulation of various phytohormones such as auxin cytokinin or ethylene (Gamalero and Glick, 2021; Santoyo et al., 2016).

Endophytic bacteria ubiquitously colonize internal tissues and facilitate plant growth by a number of different mechanisms and the functional diversity of endophytic isolates was determined by evaluating various PGP attributes. It was revealed that all the bacterial endophytic isolates (except PD26 and OC34) in our study showed significant results for PGP characteristics. There are numerous species of bacterial endophytes that flourish inside the plants and stimulate plant growth by a plethora of mechanisms involving nutrient uptake, phytohormone production as well as tolerance to abiotic and biotic stresses (Etesami et al., 2014; Hassan, 2017; Afridi et al., 2019). From the results of PGP activities, it was observed that AU15 (among all the isolated endophytes) showed best results for solubilization of phosphate with 2.3 psi. Phosphorus (P) is one of the primary nutrients essential for overall plant growth and crop productivity contributing to approximately 0.2% of the plant's dry weight (Maharajan et al., 2018; Bindraban et al., 2020). Matos et al., (2017) reported that isolate EB.78 (*Bacillus* sp.) strongly exhibited P solubilization capacity in solid medium containing Ca3(PO4)2 and soy lecithin as P sources. In another study, five endophytes namely *Pantoea vagans* IALR611, *Pseudomonas psychrotolerans* IALR632, *Bacillus subtilis* IALR1033, *Bacillus safensis* IALR1035 and *Pantoea agglomerans* IALR1325 displayed high phosphate solubilization efficiency. Further, the microbes were inoculated into different plants and it was revealed that IALR1325, IALR632, and IALR1033 significantly promoted the growth of pepper whereas IALR632 and IALR1325 increased the plant biomass of tomato plants, with a 30.5% and 22.2% increase, respectively (Mei et al., 2021).

The isolated bacterial endophytes also showed significant results for the solubilization of Zn. Zn is another essential micronutrient vital for plant growth and development and its deficiency may cause impaired integrity of cell membrane, reduced production of chlorophyll, carbohydrates, nucleotides, and cytochromes (Goteti et al., 2013). Zn solubilizing microorganisms solubilize complex zinc compounds into simpler ones and make zinc available to the plants through various mechanisms, one of which is acidification (Kamran et al., 2017). In the current study, maximum solubilization of Zn was shown by OC36 (4.26 zsi) and BN39 (3.86 zsi); though the isolate BN39 has not been found to be positive for any other PGP attributes and not chosen for further experiments. In the same context, *Bacillus megaterium* CDK25 expressed highest potential to solubilize Zn (5.0 cm) which was further confirmed quantitatively by atomic absorption spectroscopy where the isolate showed markedly higher ZnO solubilization ability (20.33 ppm) (Bhatt and Maheshwari, 2020). It was also observed that the isolate CDK25 showed a significant impact on plant growth parameters of *Capsicum annuum*, besides showing maximum zinc content in fruits (0.25 mg/100 g).

IAA production is another substantial PGP trait attributed by isolated bacterial endophytes in the present study and KA28 among all the isolates showed significant high results by producing 122.9 µg/ml of the phytohormone followed by OC36 with the value of 118.79 µg/ml. IAA is one of the most common auxins synthesized by plants playing a key role in both root and shoot development (Korver et al., 2018). It regulates various growth and developmental processes such as cell division and proliferation, tissue differentiation, apical dominance, and senescence (Bharucha et al., 2013; Nicastro et al., 2021). The auxin serves as one of the key signals playing a role in the communication between host plant and endomicrobes, and PGPE synthesizes IAA de novo to influence the IAA homeostasis in plants (Jahn et al., 2021). In the same context, endophytic strains of *B. megaterium* MJHN1 and *B. cereus* MJHN10 isolated from the nodules of *Vigna radiata* produced significantly high amount of IAA and their in vitro application increased the plant growth parameters of the host plant. The spectrophotometric analysis showed that isolate MJHN 1 produced 79.88 µg/ml at 500 µg/ml of tryptophan concentration in 24 h and MJHN10 synthesized 79.6 µg/ml of IAA at 300µg/ml in 72 h (Bhutani et al., 2018). IAA production of 11.23 ± 0.93 µM/ml by *Sphingomonas* sp. LK11 isolated from the leaves of *Tephrosia apollinea* was also evaluated in another study. Plants of tomato inoculated with this endophytic *Sphingomonas* sp. LK11 showed significantly increased growth attributes comprising shoot length, chlorophyll contents, shoot, and root dry weights with respect to control and this indicated that such phytohormones producing strains could assist in increasing crop growth as well as its productivity (Khan et al., 2014).

Iron (Fe) is an essential micronutrient required for both plant productivity as well as its nutritional quality. Plants need only a tiny amount of iron to be healthy, but that small amount is very crucial. Its deficiency is widely recognized to be one of the major problems and is manifested by yellowing of plant leaves known as interveinal chlorosis (Briat et al., 2015). Bioavailability of Fe to the plant roots is a limiting factor for the plants and is dictated by the soil redox potential and pH. Siderophores are the secondary metabolite that acts as ferric ion-specific chelators under iron stressed conditions and are secreted by most of the soil microorganisms including PGPE (Crowley, 2006; Rajkumar et al., 2010). Qualitative and quantitative estimation of the assay was performed and it was found that 69% of the endophytic isolates were found to be positive for the production of siderophores. In the present work, quantitative tests revealed that PD25 was the best siderophore producer (42.2 psu) in comparison to all other isolated bacterial endophytes. Isolate AU15 was also strongly positive for siderophore production with a value of 39.9 psu. Rungin et al., (2012) procured an endophytic *Streptomyces* sp. GMKU 3100 from roots of rice and the endophyte was positive for siderophore production on CAS agar. A siderophore-deficient (*desD*) mutant of GMKU 3100 was generated and pot culture experiments clearly showed that rice and mungbean plants inoculated with the wild type gave the best enhancement of plant growth parameters compared to untreated controls and mutants. Bacterial endophytic isolate of *Streptomyces* sp. reported prolific production of IAA and siderophores and it was also demonstrated that both played a vital role in the growth promotion of tomato plants upon inoculation (Verma et al., 2011). Antagonistic activity by *Streptomyces* endophytes against phytopathogen *Alternaria alternata* was also noticed and proposed to be linked to the high Fe complexing capacity of the endophytic isolate.

Additionally, some endophytes have the ability to control deleterious pathogens and inhibit the occurrence of diseases in plants by production of a volatile metabolite i.e., HCN. HCN is formed from glycine through the action of HCN synthetase enzyme and is known to be a powerful inhibitor of several metalloenzymes, especially copper-containing cytochrome C oxidases (Martínez-Viveros et al. 2010). There are conflicting views about the contribution of HCN producing microorganisms in plant growth and the mechanisms in which this secondary metabolite is involved (Bakker and Schippers, 1987; Etminani and Harighi, 2018). However, the production of HCN is regarded as a biocontrol mechanism involving antagonism, and some plant growth promoting microorganisms synthesizing HCN are recognized as significant biological control agents. Production of HCN increases the plant's resistance to harmful plant pathogens however little is known about the role of HCN producing endophytes in plant disease control (Senthilkumar et al., 2009; Marag and Suman, 2018). The bacterial endophytic isolates in the present study were checked for HCN production qualitatively and the results showed that none of the isolates were found to be positive for this metabolite.

Microorganisms exhibiting ACC deaminase activity are known to considerably decrease the ethylene levels in plants and facilitate plant growth under a variety of environmental stresses. ACC acts as the immediate precursor of the hormone ethylene and the enzyme ACC- deaminase cleaves ACC into α-ketobutyrate and ammonia; thereby lowering the levels of ethylene in host plants (Rashid et al., 2012). Bacterial endophytes exhibiting ACC deaminase activity are considered to be the best alternative of various chemicals to keep the stress ethylene below the growth inhibitory point (Glick, 1995; Ali et al., 2012). Characterizing the isolates for production of ACC deaminase, it was found that 8 bacterial endophytic isolates namely MP2, NA6, AU15, CP18, PD22, KA28, KA31, and OC36 were able to grow on plates containing ACC as sole N source hence confirming the presence of ACC deaminase enzyme. Various studies have exploited the role of ACC deaminase producing endophytes under stressed conditions including high salinity. Gamalero et al., (2010) reported the beneficial interaction of *Pseudomonas putida* UW4 and *Gigaspora rosea* BEG9 on the growth of cucumber plants under salt stress (72 mM) conditions. In the experiment, significant differences in plant growth parameters were observed between plants treated with wild-type *P. putida* UW4, ACC deaminase mutant bacteria compared to untreated control. Similarly, the fresh and dry mass of the canola plants treated with ACC deaminase containing *Brevibacterium epidermidis* RS15, *Micrococcus yunnanensis* RS222, and *Bacillus aryabhattai* RS341 was also observed to increase up to 47% (Siddikee et al., 2010). These reports indicate that certain halotolerant microbes have a real potential to reduce ethylene production via ACC deaminase activity and enhance plant growth under salt stressed environments.

All the obtained endophytic isolates were further screened for their nitrogen fixation activities. Isolates were preliminarily checked by growing them on N free Jenson's medium and the isolates displayed good growth while few were unable to proliferate on the respective agar medium. The medium lacks the source of N and is thereby recommended to determine the ability to fix N by various microorganisms (Chaintreuil et al., 2000; Govindarajan et al., 2008; Patel et al., 2021). According to Bhattacharjee et al., (2008) association of nitrogen fixing bacteria with several non-leguminous plants like rice, sugarcane, wheat, and maize shows a ray of hope in providing an alternative to the use of nitrogenous fertilizers whose excessive and inappropriate usage over the decades resulted into underground water leaching and greenhouse gas emissions of nitrous oxide (N_2O). Muangthong et al., (2015) followed similar screening criteria and reported nitrogenase activity for endophytic bacterial isolates C2H2L and C7HL1 closely related to *Novosphingobium sediminicola* and *Ochrobactrum intermedium*. In the present study, seven isolates i.e., AU15, CP18, PD22, PD25, KA28, KA31, OC36 with multiple PGP attributes, presence of *nifH* gene, and salt tolerance property were selected and further characterized. Likewise, Hongrittipun et al., (2014) also procured endophytic N fixing bacteria from rice plants and evaluated the effect of selected isolates i.e., *Burskholderia cepacia* (CS5), *Citrobacter* sp. (CR9), *Citrobacter* sp. (SS5), *Citrobacter* sp. (SS6), *Bacillus amyloliquefaciens* (25R14), *B. amyloliquefaciens* (SR1) and *B. thuringiensis* (25R2) on the growth of their host plant seedlings. Recently another study was also done by Rana et al., (2020) investigating the potential biotechnological applications of nitrogen fixing bacterial endophytes from diverse wheat genotypes with multifarious PGP attributes in plant growth promotion.

The selected multifarious endophytic isolates were further subjected to molecular characterization by 16S rRNA sequencing. Genotypic methods of characterization including 16S rRNA gene sequencing have been used since many years for the identification of microorganisms and are considered more authentic and reliable as compared to other phenotypic studies (Janda and Abbott 2007; de Abreu et al., 2014; Nerva et al., 2019). Phylogenetic analysis of sequenced isolates was done to characterize them at the species level and it was found that isolates belonged to diverse bacterial orders. The present study reports five bacterial genera *Bacillus, Alcaligenes, Proteus, Pseudomonas, Brevibacterium* associated with the root tissues of wheat growing in saline areas of Lucknow and adjoining regions. Among seven genetically identified isolates, two (AU15 and PD22) belonged to family Bacillaceae, and two isolates (CP18 and KA31) were found to be of family Alcaligenaceae. The remaining isolates i.e., PD25, KA28, OC36 were interpreted to be associated with the families Enterobacteriaceae, Pseudomonadaceae, Brevibacteriaceae, and identified as *P.*

mirabilis, P. marginalis, and *B. antiquum* respectively. Molecular identification of AU15, PD22, CP18, and KA31 revealed isolates as *C. firmus, P. aryabhattai*, and *A. faecalis*. The gene sequences of all the seven endophytic isolates were submitted to GenBank database of NCBI (Table 14). The two potential isolates i.e., AU15 and OC36 were submitted to the international culture collection repository at NAIMCC-Mau, India. Culture collections have become a valuable resource for the sustainable use of microbial diversity and its conservation globally and impart a vital role in the conservation and continuous use of microbial resources (Sharma and Shouche, 2014; Overmann, 2015). The newly investigated microbial taxa and strains need to be preserved to make them easily accessible to other researchers and industries. In the same regard, useful strains were submitted to the culture collection as type strains and *B. firmus* AU15 was assigned with accession number of NAIMCC-B-03040 whereas *B. antiquum* OC36 with NAIMCC-B-03041 by NAIMCC, Mau. The submitted strains are taxonomically significant as they were isolated for the first time from the root tissues of a cereal crop i.e., wheat in India. The information available on endophytic inoculants and the number of PGPE stored in different culture collection centers at the global level provides important microbiological resources to increase the usage of biological inoculants for sustainable agriculture.

C. firmus (AU15) belongs to *Cytobacillus* genus of rod-shaped bacteria stains either Gram-positive or Gram-variable within the order Bacillales and was found able to colonize the wheat plant as an efficient plant growth promoter in present study. Members of this genus were transferred from the genus of *Bacillus* after comparative genomics studies have determined that they were sufficiently different by phylogenetic measures (Gupta et al, 2020). Except for a small segment of *Bacillus* species with validly published names, all other species of *Bacillus* not related to the Subtilis or Cereus clades have been rearranged into other genera as a result of re-classification (Gupta et al., 2020). In previous reports, *B. firmus* has been studied to be associated with the plants of rice (Kaga et al. 2009), black pepper (Jasim et al, 2014), tomato (Ghahremani et al., 2020), maize (Sánchez-Bautista et al., 2018). *B. firmus* was characterized as an endophytic bacterium of peanut by and it was demonstrated that the isolate significantly enhanced the pod and haulm yield and also alleviated salinity stress in peanuts (Pal et al., 2021). Huang et al., (2021) also found colonization by *B. firmus* I-1582 in the roots of *Arabidopsis thaliana* which further significantly protected the plants from infestation by the cyst nematode *Heterodera schachtii*.

Brevibacterium is another genus that includes Gram-positive, obligate aerobe with an optimum growth temperature range of 21–28 °C. These microbes are known to be ubiquitously present in dairy products, fresh and salt water, marine organisms, insects and decaying organic matter; however interestingly few studies also report the existence of mutualistic association of *B. antiquum* with plants. *B. antiquum* AY243344 showing positive plant growth-promoting traits including siderophore capacity, phosphate solubilization, and IAA production was isolated from rhizosphere of rice and was evaluated for antagonistic potential against *M. phaseolina*. The isolate apparently well adapted the sorghum rhizospheric environment and played significant role as biocontrol agents for the control of charcoal rot in the plants (Gopalakrishnan et al., 2011). Gopalakrishnan et al., (2016) further evaluated AY243344 along with seven other bacteria for their PGP and biofortification potentials on grain legumes such as pigeon pea and chickpea. Passari et al., (2015) reported species of *Brevibacterium* showing multiple PGP traits from endophytic environments of medicinal plants. Another culturable isolate *Brevibacterium* sp. S91 showing positive results for plant growth promoting activities was reported by Borah et al., (2019).

Endophytes are a class of endosymbiotic microorganisms able to colonize and healthfully coexist with plant tissues without causing infection or negative effects on their hosts (Kloepper and Beauchamp 1992; Miguel et al., 2013). Reinfection tests were performed to test the authenticity of selected isolates as endophytes and the colonization abilities of putative endophytes inside plantlet tissues were determined. Experiments were executed in replicas in order to verify that the same bacteria inoculated to sterile wheat seeds could be re-isolated from the seedlings. The test is specific for endophytes and it was observed that the isolates re-infected the wheat plants under *in vitro* conditions which was confirmed by re-isolation (to fulfil Koch’s postulates. Though root hair cells, wounded tissues, cracks in lateral root junctions act as the key entrance points on roots for endophytic microbes, root cracks are recognized as the major ‘hot spots’ for bacterial colonization inside plants (Wahla and Shukla 2017; Singh et al., 2021). Microscopic validation is also important for the assessment of a truly endophytic lifestyle of a microorganism, thereby colonization patterns were also detected by scanning electron microscopy. Inoculated endophytic isolates inhabited the root surfaces; intercellular spaces as well as insides of root cells. Li et al.,

(2021) reported the successful colonization of *Serratia* sp. PW7 in wheat seedlings. Bacterial colonization of *Pantoea agglomerans* along with its dynamics in the wheat roots was also demonstrated by Soluch et al., (2021). Three endophytic bacterial strains i.e., BHU12, BHU16, and BHUM7 of *A. faecalis* successfully colonized plants of okra and assessed for their plant growth-promoting and antagonistic abilities (Ray et al., 2015). It has been also stated that endophytes also have certain genomic differences from rhizosphere colonizing bacteria in order to colonize the plant tissues; though no definitive group of genes responsible for endophytic lifestyle has been allocated so far (Mercado-Blanco and Lugtenberg, 2014).

The virulent nature of pathogenic microorganisms is multifactorial, but it is determined by hemolytic activity on blood agar (Mendes et al., 2013), and synthesis of exoproducts such as protease, gelatinase, and catalase (Vermelho et al. 1996). These enzymes are known to be responsible for tissue damage by degradation of collagen and proteoglycans and have been also shown to degrade proteins that are involved in host defense (Sakata et al., 1993). Proteases are enzymes that break down proteins, many of which have been implicated to play a crucial role in numerous pathological processes. A number of diseases such as inflammation, arthritis, tumor invasion, metastasis, infections, neurodegenerative and cardiac diseases have been linked with the participation of one or more proteolytic enzymes (Brown and Goldstein, 1999; Hua and Nair, 2015). Lantz and Ciborowski, (1994) stated that identification and characterization of microbial proteases are considered as prerequisites to understand their role in the pathogenesis of various infectious diseases. In the same context, *P.*

aeruginosa was tested for the production of proteases and elastases which have been implicated with the pathogenicity of the microbe (Egamberdiyeva 2005). In the present study, the endophytic isolates tested for enzymatic activities were found to be negative for protease and gelatinase activity, which suggests the avirulent and non-pathogenic nature of the microbes. Blood agar medium was used to detect and differentiate microbes on the basis of the ability to produce hemolysins (enzymes that lyse red blood cells). None of the tested isolates were found to be positive for hemolytic properties indicating towards their non-pathogenic nature; and thereby confirming the biosafety issues prior to pot experiments.

The seven selected isolates having multifarious plant growth promoting attributes were subjected to pot experiments to study PGP response on wheat. In the present study, the beneficial mutualistic associations of endophytes with the wheat plant and its productivity were explored and it was found that all the inoculated isolates significantly improved the plant growth parameters of wheat. However, among all the seven isolates two (AU15 and OC36) were found to be best plant growth promoters in boosting the growth of plant under salt stressed conditions. Elucidating the role of their endophytic inoculation, it was hypothesized that fixation of N, chelation of nutrients and salt tolerance ability by these isolates have possibly induced the growth promotion in treated plants. PGPE are previously reported to facilitate plant growth and development by a number of mechanisms including fixation of N2, solubilization of phosphate, production of indole acetic acid (IAA) and siderophores, and reduction in ethylene concentration (Glick 2012; Ryan et al., 2008). IAA production by endophytes alters the structure of roots which thereafter also restricts the uptake of sodium ions and also enhance the nutrients chelation under salt stressed conditions (Egamberdieva et al. 2019). The roots are crucial for a myriad of physiological processes and uptake of nutrients (P, Zn, Fe, and K) has also been linked to the alleviation of salinity stress in plants. Nutrients are involved in regulating various plant physiological activities, reducing uptake of sodium ions, maintaining ionic homeostasis and integrity of the cell membrane as well as modulating stomatal conductance (Keisham et al., 2018). Both the isolates i.e., AU15 and OC36 with multiple PGP characteristics not only augmented the growth and yield of wheat but also helped the wheat plants to survive in presence of salt stress. Waqas et al., (2012) observed a positive correlation between K+, Mg2+ and Ca2+ uptake by *Phoma glomerata* LWL2 and *Penicillium* sp. LWL3 and plant growth of *Cucumis* sativa under salt stress of 140mM. Additionally, the endophytic bacterium *Sphingomonas* sp. LK11 isolated from the leaves of *Tephrosia apollinea* also showed significantly increased growth attributes in tomato plants as compared to the control (Khan et al., 2014). Inoculation of rice with *Pantoea agglomerans* YS19 promoted the growth of rice and affected allocations of photosynthates as reported by Feng et al., (2006). Plant growth-promoting endophyte *Sphingomonas* sp. LK11 counteracted the salinity stress and improved the plant growth of tomato plants in normal as well as saline conditions (Khan et al., 2017b).

N is one of the most essential elements in all biological materials and serves as a main component of all proteins and enzymes, nucleic acids, and chlorophyll. Biological nitrogen fixation (BNF) has been emerged as a potentially attractive and alternative source of nitrogen for enhanced crop productivity as plants are capable of absorbing N_2 only in the form of nitrogenous compounds (ammonium, nitrites and nitrates) from the soil (Rosenblueth et al., 2018). Being an efficient N fixer, both the endophytic isolates (AU15 and OC36) may have significantly improved the uptake of N by the plants and ultimately improved the growth of wheat under greenhouse conditions. These isolates in the preliminary studies were found to actively grow on N free medium and the N fixing ability was also further confirmed with *nifH* gene and ARA analysis. Shabanamol et al., (2018) found that endophytic nitrogen fixing isolates of *Lysinibacillus sphaericus* (L1), *Klebsiella pneumoniae* (S2) and *Bacillus cereus* (R2) highly augmented the seed germination and seedling vigor of rice plants. Salinity and its interference with the nutrient availability in soil is also a very complex network that disturbs almost all the metabolic and developmental processes in plants directly as well as indirectly (Ullrich, 2002). Hence, the mutualistic relationship between the nitrogen fixing potential (detected by ARA) and salt tolerance ability of the endophytic bacterial isolates could have acted synergistically enhancing the growth parameters of plants under saline conditions. Furthermore, stress resilience in the inoculated plants could also be possibly due to the involvement of ACC deaminase enzyme produced by both AU15 and OC36 respectively. Afridi et al., (2019) stated that the ACC deaminase-producing PGPEs allied with the host plant are more capable of enduring high saline environments and enhance the plant growth efficiently. In the same context, it was earlier reported that ACC deaminase activity of *Pseudomonas* sp. UW4 played a synergistic role in protecting the tomato plants from growth inhibitory effects of high NaCl concentrations (Orozco-Mosqueda et al., 2019). Ali et al., (2014) demonstrated the role of increased resistance of tomato to salt by ACC deaminase containing bacterial endophytes (*Pseudomonas fluorescens* YsS6, *Pseudomonas migulae* 8R6) which helped the plants to grow under high salt stress condition of 165 mM and 185 mM.

Comparative population studies are deliberated to be the key for understanding endophytic diversity along with host-micro-symbiont interaction and identification of proper strain by modern molecular polyphasic approaches can disclose true diversity of the endospheric ecosystem (Kandel et al., 2017). Roy et al., (2016) surveyed the abundance of endophytes in shrubs and isolated 23 morphologically different bacteria from a medicinal shrub, *Andrographis paniculata* from West Bengal, India. Cultivable fungal and bacterial isolates (*Pseudomonas trivialis, Didymella exitialis, Alternaria infectoria, Microdochium nivale*) were explored as endogenous microbes in wheat plant of two cultivars i.e., Caphorn and Apache. Root hairs play an important role in symbiotic recognition of legumes and non-legumes especially cereals (Cocking 2003). Bacterial endophytes offer several advantages to the host plants (particularly growth promotion) comparatively to other rhizospheric occupants as they are able to communicate and interact directly with the plants in a more efficient way (Coutinho et al., 2015). This suggests that the selected endophytic isolates colonized the interiors of plants and could have directly participated in the growth promotion as a consequence of multiple PGP attributes possessed by them (fixation of N, acquisition of essential nutrients, and synthesis of ACC-deaminase). However, deeper molecular-insights are required to determine the correlation between nitrogen fixation, stress mitigating traits, and increased plant productivity.

Cereals play a pivotal role to satisfy the food demand of the growing population globally, predominantly in developing nations where cereal-based production system is the only leading source of nutritional intake (Ramadas et al., 2019). Wheat is one of the major staple food crop of nearly 2.5 billion of the world's population. Being next to rice, wheat constitutes one of the primary sources of protein intake in the least developed countries and middle-income nations (Seck et al., 2012). Salinity is the most adverse environmental factor affecting the growth and yield of wheat by causing osmotic stress and ion toxicity due to an increase in Na+ ion assimilation and decreasing Na+/K+ ratio within the plant roots (Sabagh et al., 2021). The stress delays the germination of seedlings, decreases the germination events, and seedling metabolism, causing a reduction in plant growth and crop yield (Ashraf and Foolad, 2005; Zheng et al., 2016). Comprehension of all the mechanisms (attributed by the selected isolates) together in the form of endophytic inoculants represents a very useful tool for the advancement of sustainable and more productive farming systems. Afridi et al., (2019) discussed that endophytic isolates have promising PGP attributes and can be used in order to promote the health status of soil and the growth of plants, even under saline conditions. The potential of PGPE in increasing nutrient uptake and stress resilience in plants makes them good candidates for the bio-inoculants that will help in reducing the chemical input in conventional agricultural practices (Ogale et al., 2018; ALKahtani et al., 2020). Microbes without salt tolerance properties gradually lose their PGP characteristics with an increase in NaCl concentration in salt-affected soils (Upadhyay et al., 2009; Etesami and Beattie, 2018; Ji et al., 2020). Interventions between salinity and nitrogen availability in soil is also a complex network that disturbs approximately all the processes in plant metabolism and its development. Further to mitigate salinity in plants, and show better survival along with profound nitrogen metabolism; PGPE are considered as a potential resource for saline-based agroecosystems (Jhuma et al., 2021). Although various research efforts have been made to improve the productivity of wheat but due to increased habitat loss and soil stresses, major loss of wheat plants have been observed around the globe. The present study is an attempt to explore bacterial endophytes and their PGP attributes associated to wheat plant collected from salt stressed soil of various regional districts of U.P., India. Bioformulations prepared using PGPE are a good tool to enhance the yield and quality of agricultural plants in an ecofriendly manner without causing any damage to the environment (Khare et al., 2018; Adeleke and Babalola, 2021). Endophytic inoculants have been extensively used by researchers for the growth promotion of various crops (Conn and Franco, 2004; Yang et al., 2021; Bizos et al., 2020). The symbiotic plant-endophyte interactions can also be one of the sustainable alternatives to and reclaim degraded land in saline agro-ecosystems in the near future. In search of safe alternatives of synthetic agrochemicals, the agronomists are viewing endophytes as promising plant growth promoting bio inoculants for saline agroecosystems.

The present study highlights some interesting concepts regarding application of diazotrophic PGPE and their involvement in mitigation of salt stress. Significant endophytic isolates (AU15 and OC36) with multiple PGP attributes have not only been involved in the plant growth improvement but also helped wheat to survive under salt stressed conditions. The growth parameters were statistically analyzed and a substantial increase in plant growth emphasize the direct role of endophytes in mitigation of salt stress and growth promotion. *C. firmus* identified as Gram-negative, rod-shaped flagellated plant growth promoting bacterium have been recognized as endophytes from tissues of plants but there are no reports available for its presence in the roots of wheat. Similarly, endophytic strains of *Brevibacterium* have been reported to colonize some of the plants in earlier reports; however, to the best of our knowledge no studies depict the colonization and presence of *B. antiquum* in wheat. Additionally, the role of both the isolates as PGPE in salt stressed environments is not much elucidated and very few reports defining their role in mitigation of salinity is available in literature. Procured salt tolerant bacterial endophytic isolates in this study are of significant interest for bacterial taxonomy as almost both the isolates (AU15 and OC36) are novel and have not been explored earlier from wheat plants from India or other countries of the world. Thus, the study provides insights into the occurrence and colonization of wheat plants by some novel endophytic genera and it is suggested that their application in form of bioinoculants can promote the yield of plants followed by reclamation of saline agro-ecosystems in a cost-effective, eco-friendly and sustainable manner.

CONCLUSION

Urbanization has been considered a threat to food security since it likely reduces the availability of croplands across the globe. Excessive use of chemicals in agricultural systems have raised several issues such as decreased soil fertility, reduction in soil organic matter, loss of soil carbon, and tremendous environmental problems for instance salinity and soil pollution. Abiotic stresses including soil salinity, extreme temperatures, nutrient deficiencies, drought, floods also severely affect the crop productivity by triggering a series of physiological, biochemical, morphological, and molecular fluctuations. Hence the development of effective eco-friendly cultivation strategies is need of the hour which not only improve the ecology of soil but also will lead to a sustainable and pollution-free environment. The present work provides insight into the mutualistic association of endophytes with wheat plants including identification of some novel multi-trait nitrogen fixing endophytic strains belonging to *Alcaligenes, Bacillus, Brevibacterium,* and *Proteus* genus from the root tissues of wheat from salt stressed soils of northern India. Application of nitrogen fixing and salt tolerant endophytic isolates mitigated the deleterious effects of salinity as well as played an important role in plant growth promotion of wheat grown under saline conditions. Thus, development of versatile bio-formulations involving such PGPE can remove deleterious constraints associated with the utilization of chemical formulations and can be a sustainable solution for improving crop productivity in saline agroecosystems in the near future.

SUMMARY

Soil salinity and sodicity are some of the major constraints to global cereal production that affect the expression of potential for development, growth and reproduction of the wheat plants. The presence of excess salts also interferes with nitrogen nutrition in a direct and indirect way, usually simultaneously. Nitrogen serves as a major component of amino acids (the building blocks of proteins) and chlorophyll utilized by plants for photosynthesis. The nutrient is essential to life and plays a very important role in plant growth, development, and reproduction. Microorganisms are the most natural inhabitants of diverse environments possessing enormous metabolic capabilities to mitigate various abiotic stresses such as drought, salinity, low or high temperatures. The ability to biologically fix N is limited to certain microbes which involve the representatives of various bacterial phylogenetic groups, which are collectively called as diazotrophs. Common wheat *(T. aestivum)* is a widely cultivated and significant cereal crop occupying the prime position among the ‘big three’ food crops which in India is usually cultivated in the months of October-November and harvested around April. It is typically milled into flour which is known to be used to make a wide range of foods including bread, noodles, pasta, biscuits, cakes, pastries, cereal bars, sweet and savoury snack foods, crackers, crisp-breads, sauces and confectionery. Enriched wheat flour is known to be a good source of iron, calcium, and vitamin B6 and also a decent source of several vitamins and minerals, including selenium, manganese, phosphorus, copper, and folate. In order to obviate the complexities of abiotic stress and increase the growth and yield of wheat plant, we need to recline towards sustainable agricultural alternatives such as use of PGPE in respect to plant growth promotion and mitigation of abiotic stresses. Application of endophytes in the form of bioinoculants are considered to be an eco-friendly, biotic, and safe substitute to chemical fertilizers that will maximize the productivity of *T. aestivum* without causing ecological disturbances in the soil.

In the present study, a total of 42 bacterial endophytes were isolated from the root tissues of wheat plant collected from Lucknow and adjoining districts. Plant roots exudes a lot of organic compounds which stimulates microbial growth and can have a major impact on the composition of the microbial communities. Bacterial endophytes colonizing the plants are diverse in nature and help in proper functioning of their host under abiotic as well as biotic stresses. All the isolates were characterized on the basis of morphological, physiological, biochemical and molecular basis and showed multifariousness in their size, shape, color, and growth pattern. 31 bacterial endophytic isolates were found to be Gram negative in nature and 47.61% isolates showed positive results for motility. The endophytic isolates were also found to be positive for the utilization of various carbon sources (glucose, dextrose, lactose, galactose, sucrose and maltose) and nitrogen sources (yeast extract, potassium nitrate, sodium nitrate, ammonium chloride, ammonium sulphate, and tryptophan). Further, isolates were also checked for their growth over different pH, temperatures, and salt stress conditions. It was observed that the optimum temperature of isolated endophytes was 28ºC and maximum number of isolates showed growth at ranges of pH (6 and 8). In the case of salt stress, most of the bacterial isolates were able to grow upto 2%, 4%, and 6% salt concentration whereas very few (26.1% and 9.5%) were able to tolerate 8% and 10% NaCl. Biochemical assays showed endophytic isolates to be also positive for catalase (all), amylase (50%), protease, lipase (30.9%), citrate utilization (35.7%), MR test (30.9%), VP test (21.4%), and ammonia production (all). On the basis of the biochemical properties. it was observed that the results were relatively diverse in nature and were displayed by significantly different bacterial endophytic communities. Next, the endophytic bacterial isolates were characterized for their plant growth promoting attributes and most of them were demonstrated to be positive for siderophore production (69%), IAA production (52.3%), phosphate solubilization (45.2%), zinc solubilization (30.95%), and 19% have been found positive for potassium solubilization. Whereas production of HCN was observed by none of the endophytic isolates. Qualitative ACC deaminase assay of the selected isolates was also performed and it was found that eight isolates (19%) were able to grow on the DF minimal salt medium indicating the positive test result.

All the isolates were screened for their potential to fix N by checking their growth on Jensen's medium, detecting the presence of *nifH* gene, and nitrogenase activity by acetylene reduction assay (ARA). 59.5% endophytic isolates were able to efficiently grow on N free medium and twenty-three of the total isolates (54.7%) were demonstrated to be positive for the amplification of *nifH* by yielding and end product of ~390 bp length. On the basis of the screening, seven endophytic isolates i.e., AU15, CP18, PD22, PD25, KA28, KA31, OC36 were chosen for further experimental work as the isolates showed best results for nitrogen fixation, PGP traits as well as salt tolerance.

Colonization efficiency of these selected isolates were investigated further and it was noted that the isolates were able to reinfect the roots of host plant i.e., wheat which was confirmed by re-isolation from the root tissues of treated seedlings and scanning electron microscopy. Electron micrographs of plant roots colonized by inoculated endophytes under gnotobiotic conditions were observed. No evidence of rhizospheric or endophytic colonization was seen in test control and it was interpreted that this ruled out the possibility of cross contamination.

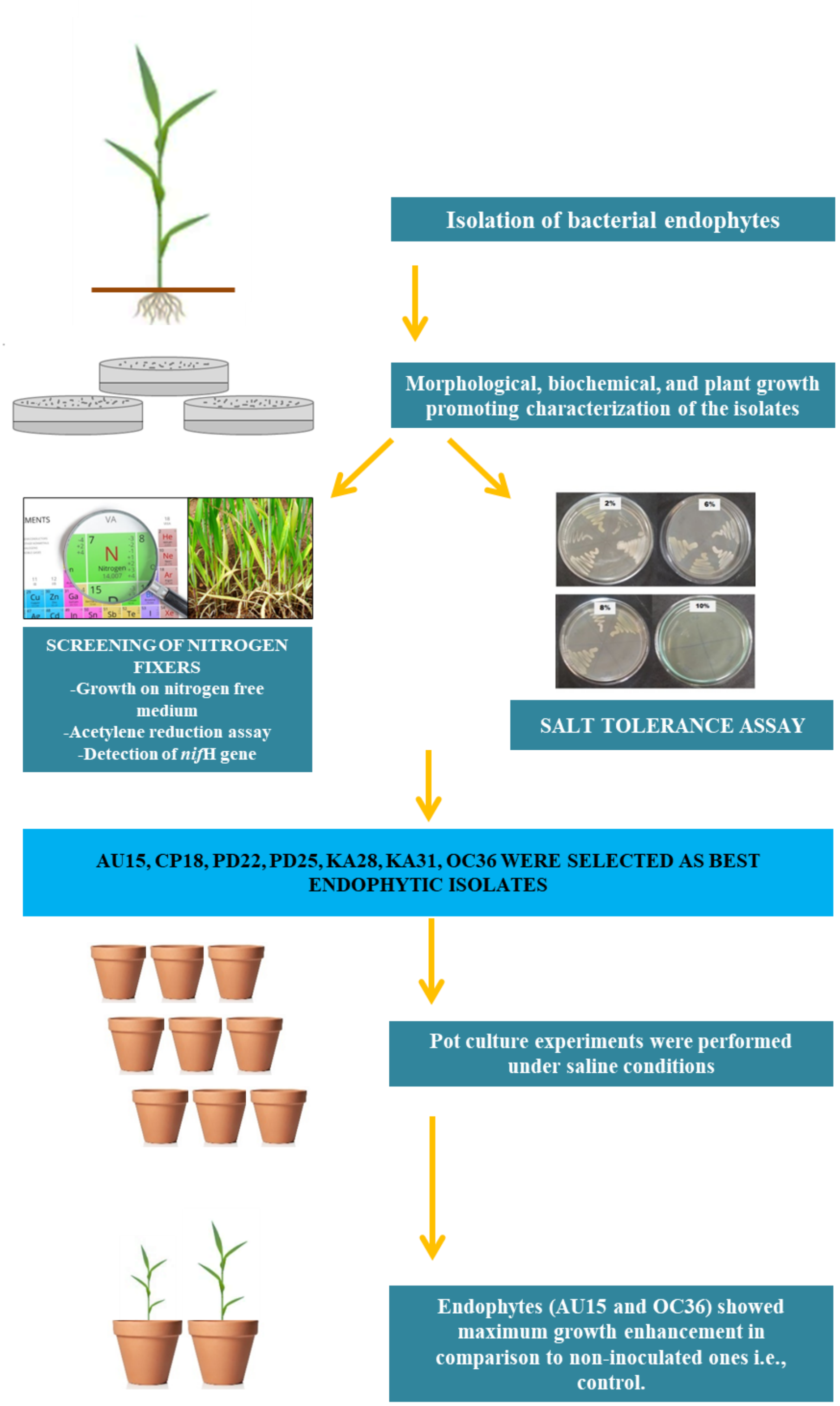
The isolates AU15, CP18, PD22, PD25, KA28, KA31, OC36 were subjected to 16S rRNA gene sequence analysis for the identification and genomic analysis confirmed them as *Bacillus firmus, Alcaligenes faecalis, Bacillus aryabhattai, Proteus mirabilis, Pseudomonas marginalis, Alcaligenes faecalis,* and *Brevibacterium antiquum* respectively. The isolates were then tested to determine biosafety issues on human health prior to the application of endophytes to the plants in pot experiments. Enzymatic activities including protease and gelatinase, hemolytic activity on hemoglobin supplemented medium, and presence of catalase, was assayed in order to determine the pathogenicity. These initial level tests proved that the isolates were non-pathogenic and avirulent; however, clinical trials will also be done to completely ensure the safety before using them at large scale.

To assess the plant growth promoting effects of the selected endophytic isolates, pot experiments were conducted for two consecutive years (2019 and 2020) using *T. aestivum* as test crop. For this wheat seeds of variety Annapurna-PBW 343 were selected and bioformulations were developed at lab scale. Sterilized seeds of wheat were treated with prepared bioformulation and sown according to different set of treatments under saline conditions (EC 9.72 dS/m). The treatment for pot study were designed as: i) Untreated control ii) Seeds + AU15 iii) Seeds + CP18 iv) Seeds + PD22 v) Seeds + PD25 vi) Seeds + KA28 vii) Seeds + KA31 viii) Seeds + OC36.

Various plant growth parameters such as root length, shoot length, plant dry weight and fresh weight, spike length, number of grains per spike and the tiller numbers were analyzed and it was noticed that seed bio priming with the endophytic isolates significantly improved growth parameters of their host plant i.e., wheat in respect to other treatments and experimental control.

Out of all the isolates applied, maximum increment was observed in the case of plant treated with two isolates namely AU15 and OC36 which were considered to be the best plant growth promoters. *C. firmus* AU15 increased the germination rate by 78.5% in comparison to experimental control while *B. antiquum* resulted into 64.2% increment. The incoulation of seeds with AU15 was the most effective treatment that caused increase in the root length by 156.1% and two-fold increment in shoot length as compared to untreated plants. Similarly, other parameters including fresh weight, dry weight, tiller numbers, spike length, and number of grains per spike were also significantly escalated by 84.5%, 293.5%, 119.7%, 88.9%, and 62.6%, respectively when AU15 was applied. The two potential isolates were deposited in National Agriculturally Important Microbial Culture Collection (NAIMCC), an international culture collection centre approved by International Depository Authority (IDA) and assigned with accession number *B. firmus* NAIMCC-B-03040 (AU15) and *B. antiquum* NAIMCC-B-03041 (OC36).

The present work elucidates the mutualistic associations of potent diazotrophic salt tolerant endophytes such as *C. firmus* AU15 and *B. antiquum* OC36 which not only played an important role in plant growth promotion of wheat plants but also mitigated the detrimental effects of salinity. Though *C. firmus* and *P. aryabhattai* have been recognized as endophytes from a variety of plants but their role in nitrogen fixation and salt tolerance in wheat plants has not been studied yet. The procured endophytic isolates are of significant interest for bacterial taxonomy and thereby development of versatile bio-formulations using these PGPE could remove the deleterious constraints associated with the utilization of chemical formulations and be a sustainable solution for improving crop productivity of wheat in saline agroecosystems in the near future.



Isolation of bacterial endophytes

Morphological, biochemical, and plant growth promoting characterization of the isolates

SCREENING OF NITROGEN FIXERS
-Growth on nitrogen free medium
-Acetylene reduction assay
-Detection of *nif*H gene

SALT TOLERANCE ASSAY

AU15, CP18, PD22, PD25, KA28, KA31, OC36 WERE SELECTED AS BEST ENDOPHYTIC ISOLATES

Pot culture experiments were performed under saline conditions

Endophytes (AU15 and OC36) showed maximum growth enhancement in comparison to non-inoculated ones i.e., control.

Fig. 32: Summary of the work done

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APPENDIX

NUTRIENT AGAR-

Ingredients	**gms/liter**
Beef extract	3.0 gm
Peptone	5.0 gm
Sodium chloride	8.0 gm
Agar	15.0 gm
Distilled water	1000 mL

YEAST EXTRACT MANNITOL AGAR-

Ingredients	**gms/liter**
Yeast extract	1
Mannitol	10
Dipotassium phosphate	0.5
Magnesium sulphate	0.2
Sodium chloride	0.1
Agar	20 g

NUTRIENT BROTH-

Ingredients	**gms/liter**
Beef extract	1.5 gm
Yeast extract	1.5
Peptone	5.0 gm
Sodium chloride	5.0 gm
Distilled water	1000 mL

MOTILITY TEST MEDIUM-

Ingredients	**gms/liter**
Tryptose	10
Sodium chloride	5.0 gm
Agar	5

TRYPTONE WATER (TRYPTONE BROTH)-

Ingredients	**gms/liter**

Tryptone	10.0
Sodium chloride	5.0
Calcium chloride- Previously sterilized $CaCl_2$ was added after autoclaving	1.0ml
Distilled water	1000ml

TRYPTONE WATER (TRYPTONE BROTH)-

Ingredients	**gms/liter**
Tryptone	10.0
Sodium chloride	5.0
Calcium chloride- Previously sterilized $CaCl_2$ was added after autoclaving	1.0ml
Distilled water	1000ml

PEPTONE WATER (PEPTONE BROTH)–

Ingredients	**gms/liter**
Peptone	10.0
Sodium chloride	5.0

SIMMON’S CITRATE AGAR –

Ingredients	**gms/liter**
Ammonium dihydrogen phosphate	1.0
Dipotassium hydrogen phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Magnesium sulphate	0.2
Bromothymol blue	0.08
Agar	20.00
Distilled water	1000 ml

MR-VP BROTH –

Ingredients	**gms/liter**
Peptone	7.0

Potassium phosphate	5.0
Dextrose	5.0
Distilled water	1000.00 ml

TRYPTONE YEAST EXTRACT BROTH –

Ingredients	**gms/liter**
Tryptone	5
Yeast extract	3
Distilled water	1000.00 ml

UREA AGAR –

Ingredients	**gms/liter**
Peptone	1.0
Sodium chloride	5.0
Potassium monohydrogen (or dihydrogen phosphate) phosphate	2.0
Glucose*	1.0
Phenol red solution**	6.0 ml
Urea (20%aqueous solution)	100 ml
Agar	20.00
Distilled water	1000 ml

*Add glucose and phenol red to the molten base and steam for 1 hour, cool to 50°C.

**Add 100 ml urea (filter standardized solution) to the basal medium.

STARCH AGAR-

Ingredients	**gms/liter**
Starch	20.0
Peptone	5.0
Beef Extract	3.0
Agar	15.0
Distilled water	1000ml

SKIM MILK AGAR-

Ingredients	**gms/liter**
Skim milk powder	100
Peptone	5.0
Agar	15.0

PIKOVSKAYA’S AGAR –

Ingredients	**gms/liter**
Yeast extract	0.5
Dextrose	10.0
Calcium phosphate	5.0
Ammonium sulphate	0.5
Potassium chloride	0.2
Magnesium sulphate	0.1
Magnesium sulphate	0.0001
Ferrous sulphate	0.0001
Agar	20
Distilled water	1000 ml

ALEKSANDROW AGAR-

Ingredients	**gms/liter**
Magnesium sulphate	0.5
Calcium carbonate	0.1
Potassium alumino silicate	2.0
Dextrose (Glucose)	5
Ferric chloride	0.005
Calcium phosphate	2
Agar	20
Distilled water	1000 ml

POTATO DEXTROSE AGAR-

Ingredients	**gms/liter**
Potatoes, infusion from	200
Dextrose	20
Agar	15
Distilled water	1000 ml

ALEKSANDROW AGAR-

Ingredients	**gms/liter**
Magnesium sulphate	0.5
Calcium carbonate	0.1
Potassium alumino silicate	2.0
Dextrose (Glucose)	5
Ferric chloride	0.005
Calcium phosphate	2
Agar	20
Distilled water	1000 ml

JENSEN'S MEDIUM-

Ingredients	**gms/liter**
Sucrose	20
Dipotassium hydrogen phosphate	1
Magnesium sulphate	0.5
Sodium chloride	0.5
Ferrous sulphate	0.1
Sodium molybdate	0.005
Calcium carbonate	2
Agar	15
Distilled water	1000 ml

STAINS, INDICATORS, AND REAGENTS

a) Gram staining

Crystal violet solution:

Dissolved 2.0 gm crystal violet in 20.0 ml of 95% ethyl alcohol

Gram's Iodine solution:

Mixed 1.0 gm Iodine, 2.0 gm potassium iodide in 300.0 ml of distilledwater

Safranin:

Dissolved 10.0 ml of safranin into 100.0 ml of distilled water

b) Indole production test

Kovac's reagent:

Dissolved the 5.0 gm of diaminobenzaldehyde in the 75.0 ml of amyl alcohol. Thenadd 25.0 ml of hydrochloric acid to the above preparation. Store the reagent in the refrigerator.

c) MR-VP test

Methyl red indicator:

Dissolved methyl red (0.1 gm) in 500 ml of 95% ethyl alcohol. Add distilled water and filter the preparation.

VP reagent I:

5.0 gm alpha-naphthol was weighed and dissolved in 95.0 ml of absolute ethyl alcohol.

VP reagent II:

40% potassium hydroxide (KOH)

d) IAA production

Salkowski reagent: 2 ml 0.5M $FeCl_3$ and 49ml water and 49 ml 70% perchloric acid.

e) Ammonia production

Nessler's reagent : Dissolve 50.0 gm of potassium iodide in 35 ml of distilled water and added saturated solution of mercuric chloride. Added 400 ml of potassium hydroxide. Dilute to 1000 ml byaddition of distilled water. Allow to settle for one week. Stored in tightly stopper brown bottles.

f) HCN production

2% Sodium carbonate solution: Dissolve 2 gm of $NaCO_3$ in 100 ml of distilled water.0.5% Picric acid solution Mix 0.5 gm picric acid in 100 ml of water.

g) Amylase production

Gram’s Iodine: 1.0 gm Iodine and 2.9 gm of Potassium iodide was mixed and add water to make total of 300 ml.

h) Cellulase production

Congo red dye (1 mg/1 ml solution) is used for staining. 1M NaCl solution is used for destaining

PUBLICATIONS

Publications

Research articles:

1. Arora NK, Fatima T, Mishra J, Mishra I, Verma S et al. (2020) Halo-tolerant plant growth promoting rhizobacteria for improving productivity and remediation of saline soils. Journal of Advanced Research 26: 69-82. DOI: 10.1016/j.jare.2020.07.003 (IF: 10.479)
2. Verma S, Verma R, Verma P, Bharti C, Arora NK (2021) Salt tolerant endophytic and diazotrophic strain of *Proteus mirabilis* PD25 and its effect on the growth of wheat under saline conditions. NVEO-Natural Volatiles & Essential Oils Journal 13172-13183.
3. Verma M, Verma S, Arora NK (2018) Application of Rhizobium-Pseudomonas consortia for enhanced production of mungbean in sustainable manner. International Journal of Science Technology and Society 3: 54-61.

Book Chapters:

1. Arora NK, Fatima T, Mishra I, Verma S (2020) Microbe-based Inoculants: Role in Next Green Revolution. In: Shukla V, Kumar N (eds) Environmental Concerns and Sustainable Development. Springer, Singapore, pp. 191-246. DOI: 10.1007/978-981-13-6358-0_9

2. Fatima T, Verma P, Verma S, Alaylar B, Arora NK (2022) Role of Metabolites Produced by Plant Growth-Promoting Bacteria in Biocontrol of Phytopathogens Under Saline Conditions. In: Arora NK, Bouizgarne B (eds) Microbial BioTechnology for Sustainable Agriculture Volume 1. Springer, Singapore, pp. 287-324

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Halo-tolerant plant growth promoting rhizobacteria for improving productivity and remediation of saline soils

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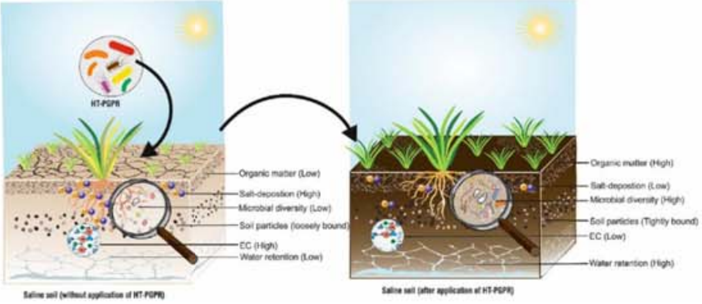
GRAPHICAL ABSTRACT



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ABSTRACT

Background: The collective impact of climate change and soil salinity is continuously increasing the degraded lands across the globe, bringing agricultural productivity and food security under stress. The high concentration of salts in saline soils impose osmotic, ionic, oxidative and water stress in plants. Biological solutions can be the most reliable and sustainable approach to ensure food security and limit the use of agro-chemicals.

Aim of Review: Halo-tolerant plant growth promoting rhizobacteria (HT-PGPR) are emerging as efficient biological tools to mitigate the toxic effects of high salt concentrations and improve the growth of plants, simultaneously remediating the degraded saline soils. The review explains the role of HT-PGPR in mitigating the salinity stress in plants through diverse mechanisms and concurrently leading to improvement of soil quality.

Key Scientific Concepts of Review: HT-PGPR are involved in alleviating the salinity stress in plants through a number of mechanisms evoking multipronged physiological, biochemical and molecular responses. These include changes in expression of defense-related proteins, exopolysaccharides synthesis, activation of antioxidant machinery, accumulation of osmolytes, maintaining the Na^+ kinetics and improving the levels of phytohormones and nutrient uptake in plants. The modification of signaling by HT-PGPR inoculation under stress conditions elicits induced systemic resistance in plants which further prepares them against salinity stress. The role of microbial-mechanisms in remediating the saline soil through structural and compositional improvements is also important. Development of novel bioinoculants for saline soils

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Salt tolerant endophytic and diazotrophic strain of *Proteus mirabilis* PD25 and its effect on the growth of wheat under saline conditions

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Abstract

In the present investigation, an endophytic diazotroph PD25 with novel properties was isolated from roots of wheat growing in saline soil (EC~10.6 dS m^{-1}). The isolate was chosen for further experimental work based on plant growth promoting (PGP) attributes and identified as *Proteus mirabilis* by 16S rRNA sequence homology. The work presented in this article reports the salt tolerant bacterium *P. mirabilis* PD25 as effective diazotrophic endophyte and the isolate was also found to be positive for other PGP attributes such as siderophore, indole acetic acid (IAA), accompanied by the solubilization of phosphorus (P), zinc (Zn), and potassium (K). The data signifies the prominent involvement of plant growth promoting endophyte (PGPE) in agriculture and suggests that novel bioformulations developed by them can be a potential development strategy in boosting plant growth and development in saline soils in the future.

Keywords: Plant growth promoting endophytes, Wheat, Bioformulations, Soil salinity, *Proteus,* Nitrogen fixation.

1. Introduction

The effects of climate change on the environment have been very significant over the years and have caused various abiotic stresses, negatively affecting crop quality and productivity. Salt stress is one of the major abiotic threat to agriculture that has impacted yield of plants in many areas of the world due to increased use quality of water for irrigation and soil salinization [1]. Reduced growth of roots and shoots, slow germination rate, decreased or no development of seedling, stomatal closure, and deterioration of photosynthetic activity are some of the key responses of plants to stress [2, 3]. Nitrogen metabolism and phytohormones production play significant roles in improvisation of growth and yield of various plants and is regulated an complex enzyme known as nitrogenase [4]. Nitrogenase is mainly responsible for biological nitrogen fixation (BNF) and synthesis is regulated by oxygen (O_2) and ammonia (NH_3) [5]. Microorganisms present in soil are able to fix nitrogen from the atmosphere with the help of various genes reported to be involved in the process of nitrogen fixation and most commonly reported genes for synthesis of nitrogenase comprise of *nifH*, *nifD*, and *nifK* [6]. Endophytes are the microbial endosymbionts including bacteria, fungi, and actinomycetes that reside in the microenvironment of the host plant [7]. Diazotrophic endophytes possessing the nitrogen fixing genes play beneficial role in increasing uptake by plants which further results in promotion of various attributes ultimately improving the health and productivity of plants. Thereby this work was conducted to study the effect of salt tolerant bacterial endophytes on growth and vigor index of one of the most important cereal crop i.e, wheat in saline soils along with the assessment of their potential of nitrogen fixation in such condition. Understanding diversity of these beneficial endophytes and their application as bioinoculants in agricultural sector can be a suitable bio-approach to reclaim salt-stressed soils and increase the plant biomass production of wheat and other cereal crops.

RESEARCH ARTICLE

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Application of *Rhizobium-Pseudomonas* consortia for enhanced production of mungbean in sustainable manner

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ABSTRACT

In this study plant growth promoting rhizobacteria (PGPR) were isolated from root nodules of *Vigna radiata* and rhizospheric soil of *Abrus precatorius* respectively. On the basis of morphological, biochemical and physiological characterization, isolate R5 (from root nodules of *V. radiata*) was identified as *Rhizobium* and P21 (from rhizosphere of *A. precatorius*) as *Pseudomonas*. Isolates R5 and P21 showed positive results for phosphate solubilization, zinc solubilization, nitrogen fixation, production of indole acetic acid (IAA), siderophore and exopolysaccharides (EPS). Isolates (R5 and P21) were applied as coinoculant on mungbean (*V. radiata*) to check their impact on its growth in laboratory conditions and pot trials. Seed germination rate and growth analysis of plants were observed by measuring growth parameters (root length, shoot length, leaves count, root nodule count, fresh weight, dry weight and chlorophyll content). It was observed that R5 and P21 significantly enhanced the plant growth in comparison to control (without treatment), but consortial inoculation was more effective in comparison to mono-inoculation of R5 and P21.

INTRODUCTION

Leguminosae is economically and ecologically very important family of Kingdom Plantae (Harborne, 1994). Legumes are the second most important food crops for world agriculture (ILDIS, 2006). Mungbean is a significant pulse crop with high nutritive value and used as a world's major source of food (Yadav *et al.,* 2014). India is the largest producer and consumer of mungbean and accounts for about 65% of the world acreage and 54% of the world production of this crop (Lambrides *et al*., 2007; Sehrawat *et al*., 2014). Although mungbean is required on large scale but due to some agro-ecological conditions, the nodulation of mungbean is very poor, which causes lower yields (Ahmed *et al.,* 2006). The excessive uses of chemical fertilizers are used for higher yield, but this approach has several negative implications on our environment (Arora *et al*., 2012; Hosseini *et al.,* 2014). The application of PGPR as biofertilizers causes a beneficial and cost effective strategy in growth enhancement (Mayak *et al.,* 2004; Mishra and Arora, 2016; Arora *et al*., 2017).

PGPR impart a great agronomic importance and influence plant development directly by producing metabolites such as plant growth regulators (hormones and other metabolites), siderophore production, phosphate solubilisation and symbiotic nitrogen (N_2) fixation and indirectly through modification to the activity of other plant-microbe interactions or by inducing changes in the microbial population balance, for instance, exerting biological control against plant pathogens (Tewari and Arora, 2013; Ahemad and Kibret, 2014; Vejan *et al*., 2016; Mishra *et al.,* 2017). The root nodulating bacteria (rhizobia) are well known for their symbiotic association with legumes mainly for biological nitrogen fixation (BNF) (Sessitsch *et al.,* 2002) and used for sustainable crop production (Laranjo *et al.,* 2014; Gopalakrishnan *et al.,* 2015; Arora *et al.,* 2017). One of the most promising groups of bacteria amongst PGPR are *Pseudomonas,* which also have various beneficial plant growth promoting traits (Moeinzadeh *et al.,* 2010; Mayz *et al.,* 2013; Tewari and Arora 2016; Mishra *et al*., 2017). Currently applications of various PGPR as co-inoculant are growing as a very beneficial trend in sustainable agriculture for higher crop yield.

Co-inoculation of PGPR especially *Pseudomonas* with *Rhizobium* is visualized as an important practice in the development of sustainable agriculture (Singh *et al.,* 2013; Arora *et al*., 2014). Co-inoculation improves plant growth by affecting some physiological functions such as by reduction in ethylene level (Shaharoona *et al.,* 2006), direct

Microbe-based Inoculants: Role in Next Green Revolution

9

Naveen Kumar Arora, Tahmish Fatima, Isha Mishra,
and Sushma Verma

Abstract

Increasing food demand, with growing population, has been a major concern throughout the globe. The aim can only be achieved with the onset of next green revolution being much defined by sustainable approaches. The past green revolution had its negative impact due to excessive use of agrochemicals contaminating the environment and further challenging the food security. Henceforth, designing the blueprint of next green revolution requires the application of effective and sustainable approaches which enhance the yield of plants while still maintaining the decorum of sustainability. In this regard, microbes have been concluded as the best players finding their roles in plant growth promotion and also stress management. Currently, there are several bacterial-, fungal-based inoculants available in the market along with genetically modified organisms, forming the base of upcoming green revolution. Thus, the future of sustainable agriculture is related to the efficiency and action of these microbes.

Keywords

Microbial inoculants · Green revolution · Environmental sustainability · Plant growth-promoting rhizobacteria (PGPR) · Stress

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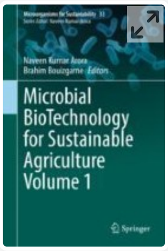

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Role of Metabolites Produced by Plant Growth-Promoting Bacteria in Biocontrol of Phytopathogens Under Saline Conditions

Tahmish Fatima, Priyanka Verma, Sushma Verma, Burak Alaylar & Naveen Kumar Arora

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154 Accesses

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Abstract

Soil salinity has continuously degraded the quality and quantity of crops and has challenged their health and defense mechanisms. Although relationship between salinity and biotic stress is poorly understood, yet due to negative impact of salts on plants, they become susceptible to diseases. Limiting the use of agro-chemicals, salt-tolerant plant growth-promoting rhizobacteria (ST-PGPR) are emerging as potential replacements for use against combinatorial stresses such as salinity and pathogens. Under combinatorial stress, i.e., salt