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RESEARCH ARTICLE

SCREENING OF GROWTH PROMOTING ENTOPHYTIC BACTERIA FOR SALINITY STRESS TOLERANCE

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ABSTRACT

Saline soil is characterized by a high concentration of salts, primarily sodium chloride. It is commonly found in dry and semiarid regions, coastal areas, and locations with elevated salt levels in the parent material. The presence of salinity in soil can have detrimental effects on plant growth, as the excessive salt content interferes with the plant's ability to absorb water and nutrients. This osmotic imbalance makes it challenging for plants to extract water from the soil, negatively impacting their growth. In Pakistan, salinity poses a significant challenge in agriculture, affecting approximately 6.6 million hectares of land. To address this issue, a study was conducted with the main objectives of identifying endophytic bacteria and determining their threshold level of tolerance to high salinity stress. The researchers employed various techniques, including the isolation of endophytic bacteria on L.B media plates, morphological assessments of bacteria, salt tolerance isolation techniques, and biochemical methods.The results of the study revealed that the endophytic bacteria isolated could withstand salinity stress levels of up to 1700mM, which represents their maximum capacity to handle such conditions. After 24 hours of incubation, changes in colony color, diameter, status, and appearance were observed. The biochemical analysis demonstrated positive catalase activity, indicated by the formation of bubbles in a liquid form, as well as a change in the color of the solution.In conclusion, the study highlights the potential of endophytic bacteria as beneficial tools for enhancing plant.

KEYWORDS

Saline soil,Endophytic bacteriaHigh salt stress tolerance,Plant growth improvement.

1. INTRODUCTION

Saline soil has a high concentration of salts, particularly sodium chloride. Salinity in the soil can occur naturally in arid and semiarid regions, coastal areas, or regions with high salt levels in the parent material (Ali Harivandi et al., 2015). It can also be caused by human activities such as excessive use of fertilizers, irrigation with salty water, and improper disposal of industrial effluent (Akhtar et al., 2021). The salt accumulation in the soil can occur due to the natural process of evapotranspiration, where water evaporates from the soil surface and leaves behind dissolved salts. This process can lead to salt accumulation in the soil in areas with low rainfall and high temperatures. In addition, using irrigation water containing high salt levels can exacerbate the problem by adding more salt to the soil. (Salama et al., 1999).

Saline soils can negatively affect plant growth, as the high concentration of salts interferes with the plant's ability to take up water and nutrients. The high salt levels create an osmotic imbalance, making it harder for the plant to absorb water from the soil. As a result, plants growing in saline soils may exhibit stunted growth, wilting, leaf burn, and reduced yields. (Applied and 2012, n.d.)

Salinity can also impact soil structure by causing soil particles to bind together, reducing soil permeability and increasing erosion risk. In addition, high salt levels can reduce soil fertility by disrupting the balance of essential nutrients and making them unavailable to plants. Hailu et al., 2021). Endophytic bacteria are a group of microorganisms that live within the tissues of plants without causing harm to the host plant (Zinniel et al., 2002). These bacteria have been found to have a range of beneficial effects on plant growth and health, including in saline soils.

Saline soils are characterized by high salt levels, which can lead to osmotic stress in plants. (García-Mauriño et al., 2003). Saline soils are characterized by high salt levels, which can lead to osmotic stress in plants (García-Mauriño et al., 2003). This stress can affect plant growth, reduce crop yields, and cause other negative impacts on plant health. However, some endophytic bacteria have been found to improve plant tolerance to salt stress and promote growth in saline soils (Vaishnav et al., 2019).

Endophytic bacteria can help to improve plant tolerance to salt stress by producing compounds that act as osmoprotectants (Kumar et al., 2020). These compounds can help to maintain cellular function and integrity under high salt conditions. For example, some endophytic bacteria produce compatible solutes such as trehalose, which help to regulate the plant's water balance and protect the plant against salt stress.

Salinity is a major problem in agriculture in Pakistan, where an estimated 6.6 million hectares of land is affected by salinity (Umali, 1993). This has negative impacts on crop yields and farmers livelihoods. However,

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research on using endophytic bacteria to improve plant growth and tolerance to salt stress in saline soils is relatively new in Pakistan.

Several studies have investigated the effect of endophytic bacteria on plants grown in saline soils in Pakistan (Shah et al., n.d.). For example, one study investigated the effect of the endophytic bacterium *Bacillus cereus* on the growth and yield of rice under saline conditions. The study found that inoculation with the bacterium improved rice growth and yield under both low and high salt conditions, likely due to the production of osmoprotectants by the bacterium. (Jha et al., 2011).

Another study investigated endophytic bacteria's effect on wheat growth and yield under saline conditions. The study found that inoculation with the endophytic bacterium *Pseudomonas aeruginosa* significantly improved wheat growth and yield under saline conditions. (Kumawat et al., n.d.). The bacterium was found to produce osmoprotectants and enzymes that break down salt ions and make them more available to the plant.

In addition to these studies, researchers in Pakistan have also investigated the diversity of endophytic bacteria in plants grown in saline soils. (Szymańska et al., 2018). This suggests that endophytic bacteria may play an important role in plant adaptation to saline soils in Pakistan. (Pirhadi et al., n.d.). Overall, research on the use of endophytic bacteria in saline soils in Pakistan is still in its early stages. However, the studies conducted so far suggest that endophytic bacteria have the potential to improve plant growth and yield under salt stress in Pakistan, which could help to address the problem of salinity in agriculture (Zahir et al., 2019). Further research is needed to identify and characterize endophytic bacteria with beneficial properties in Pakistan and develop strategies for their application in agriculture.

Therefore, we investigate the saline soil of Karak District, KpK Province, Pakistan. The main objectives of this study are to identify the endophytic bacteria and their threshold level of high salinity stress tolerance. This study gives a guideline for determining salinity and addresses the salinity problem in agricultural land.

2. Materials and Methods

2.1 Study area and Sampling Collection

Soil samples were collected from Karak District, KpK Province, Pakistan (Figure 1). soil samples were collected from three different depths from two separate sites. The dept differences on each site are 0-5, 5-10, and 10-15 cm soil depth. All the samples were collected in sealed sterile plastic bags and transported aseptically to the laboratory.

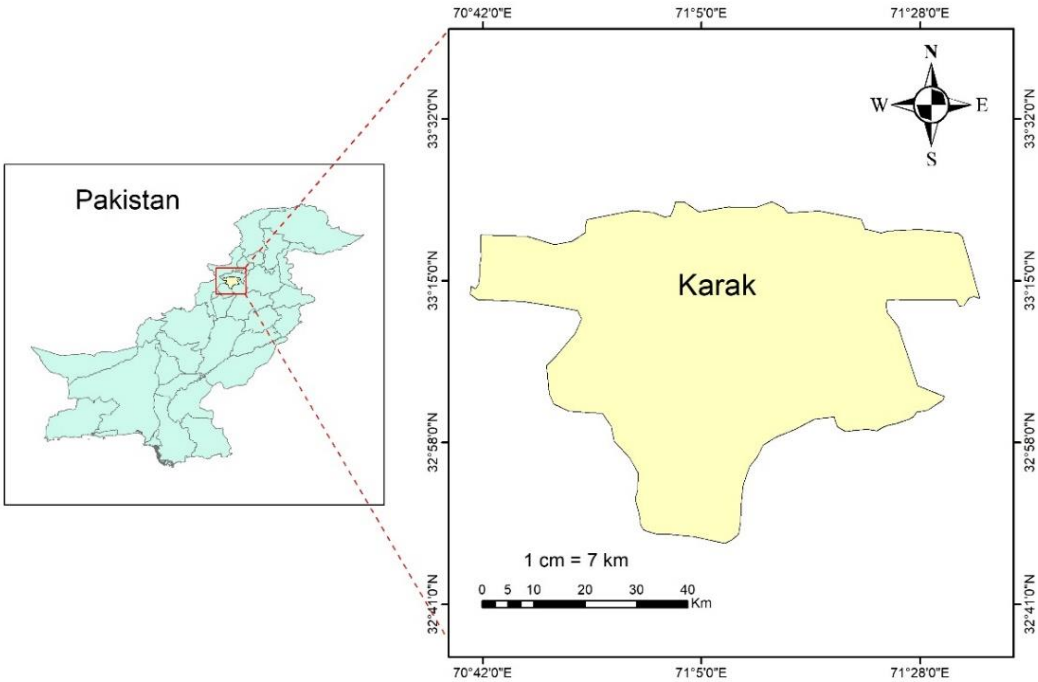

Figure 1: Location map of Study area (District Karak,Kohat,Pakistan)

2.2 Preparation of Bacteria Inocula

To perform each of the tests in this study, the fresh inoculum of each isolate was prepared in the following way. Initially, endophytic bacterial isolates were re–cultured on an LB medium. The 100 mL flask containing 20 mL of sterilized LB culture medium were inoculated with bacterial isolates. The cultures were incubated at 37°C for 48 h (for fast– growing bacteria) and 72 h (for slow–growing bacteria) on a shaker with a rotation speed of 120 rpm until (turbid). The bacterial cells were harvested through centrifugation (7000 g for 10 min) after growth in LB culture medium, washed twice with phosphate buffer, and then suspended in the same buffer.

2.3 Morphological Characterization

Bacteria were grown, but changes in colour, diameter, status and appearance of colonies were investigated after 24 hours of incubation. The tolerance of isolates to different salinity levels was evaluated by observing the quality of the colonies grown in the control plates. For different concentrations of salinity stress 50 mM, we can add 50 mM NaCl concentration in the LB media. Then we can streak bacteria grow, and this process continues, the salt concentration up to 1800 mM.

2.4 Salt Tolerance Test of Isolates

For this purpose, NA medium containing different salinity percentages (50 to 1800 mM) NaCl salt) was used. At first, culture media, including various concentrations of NaCl, were distributed in sterile conditions in Petri dishes. The fresh culture of each isolate with a uniform population was spotted on the agar plates. For each isolate, three replicates were taken, and the petri dishes were kept in the incubator at 37°C after sealing.

2.5 Biochemical Characterization

2.5.1 Catalase Test

Catalase activity of the isolates was estimated by using an H_2O_2 solution on the microscopic slides containing the culture of the isolates separately. Observations were taken for immediate bubble formation (O_2 + water = bubbles). The release of O_2 bubbles indicated positive catalase activity. It is used to differentiate *staphylococci* (positive) from *streptococci* (negative). The enzyme catalase is produced by bacteria that respire using oxygen and protect themselves from the toxic byproduct of oxygen metabolism. We took six samples and dropped a few drops of H_2O_2 on each plate using a dropper. As a result, bubbles like liquid form, showing positive catalase activity.

2.5.2 Nessler's Reagent Test

An aqueous solution of potassium iodide (7.0gm), mercuric chloride (10.0gm), and sodium hydroxide is used to determine the presence of ammonia in a specific material. When these reagents detect ammonia in a

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particular substance, the color of the solution will change to yellow. First, the Nessler reagent was formed and then poured into six falcon tubes, each with five microliters. Then colonies of endophytic bacteria were picked using a toothpick and dropped in each of the six falcon tubes. After that, we kept these falcon tubes in a continuous shaker at 37°C for 12 hours. The next day the solution of color was changed, which showed positive results.

3. Results and Discussion

3.1 Salt tolerance test of bacterial isolates

3.1.1 Isolated endophytic bacteria were tolerant to Salinity stress (50mM to 200mM):

Endophytic bacteria were isolated from maiz crops roots .First its plant growtg promoting activities were analyzed by checking its tests for IAA production in order to know wether these bacterias has a role in salinity stress. These endophytic bacteria were exposed to different concentrations of salinity stresses 50mM,100mM,150 mM,and 200 mM stress of NaCl on LB respectively each; experiment was continue after successful growth of these bacteria on LB and salt stress media after growth on 1 plate of salt stress the level for salanity were evaluated gradually it was noted that the salt stress upto 200mM didn't inhibit the growth of these endophytic bacteria suggesting that these endophytic bacteria a role of salinity stress .Isolate were known to have different level of maximum salt concentration stress tolerance for the growth and yield. But some bacteria were growing upto 200mM stress of Nacl but showing the same results and grow on plates as same as 50mM stress of Nacl. The temperature and various morphology of bacteria were recorded for each isolate Morphology were help in identification of streptococcus and staphylococcus. For example pseudomonas family. It is confirm that the bacteria were capable of growing to tolerate the salinity stress.

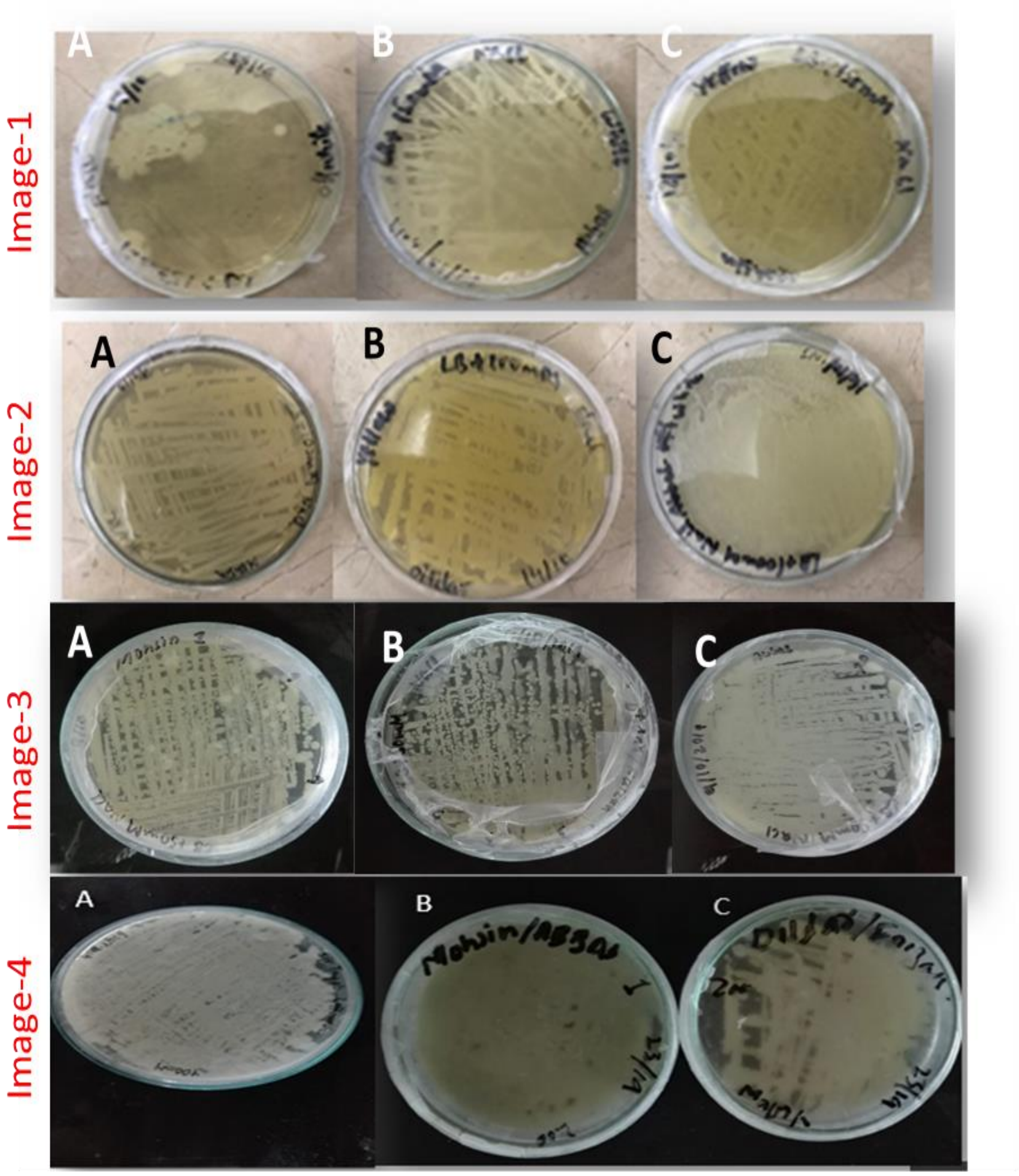

Figure 2: Growth of endophytic bacteria from 50mM to 200 Nacl stress

Image 1: Show 50 mM Nacl Stress.Image 2: Show 100 mM Nacl Stress.

Image 3: Show 150 mM Nacl Stress.Image 4: Show 200 mM Nacl Stress.

3.1.2 Isolated endophytic bacteria were tolerant to Salinity stress (400mM to 1000mM)

To analyze that the level of the endophytic bacteria can cope with the salinity stress a second round of salinity stress were given. The endophytic bacteria were exposed to different stresses after growing in one stress and the level of stress were elevated gradually. On exposure to 400mM to 1000mM it was observed that the endophytic bacteria can grow at any of these stresses without inhibition in their normal routine growth at 37 C, suggesting that these bacteria's are capable of handling salinity stress regardless of its growth promoting activities as shown in figure 3 surprisingly there was no reduction at the growth rate of these bacteria.

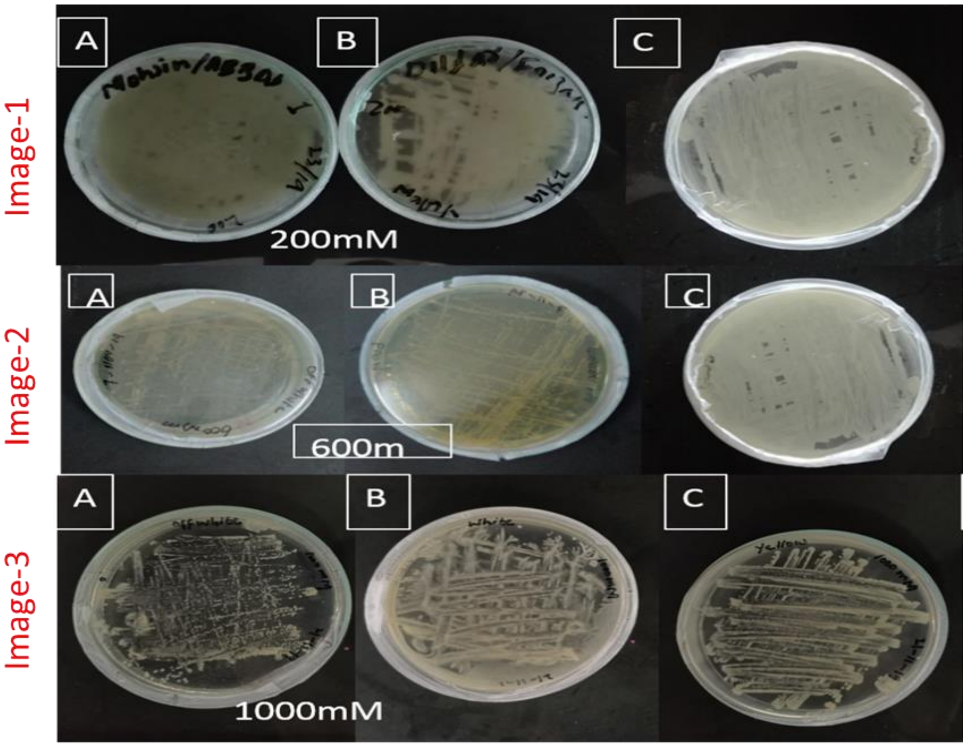

Figure 3: Growth of endophytic bacteria from 400mM to 1000mM NaCl stress

Image 1: Show 200 mM Nacl Stress,Image 2: Show 600 mM Nacl Stress.

Image 3: Show 1000 mM Nacl Stress

3.1.3 Isolated endophytic bacteria screening for maximum salinity stress threshold level

In order to characterize what's the maximum threshold level for salinity stress. Isolated endophytic bacteria were exposed to 1200mM, followed by 1500mM, 1650mM and 1800mM. The data suggested that these bacteria were resistance to 1200mM, 1500m, and 1650mM. However at 1800mM these bacteria did not show any growth hence no colonies were seen on the plates as shown in figure 3. These results confirmed that these bacteria can handle salinity stress upto 1700mM which is the maximum capacity of these endophytic bacteria to handle this situation.

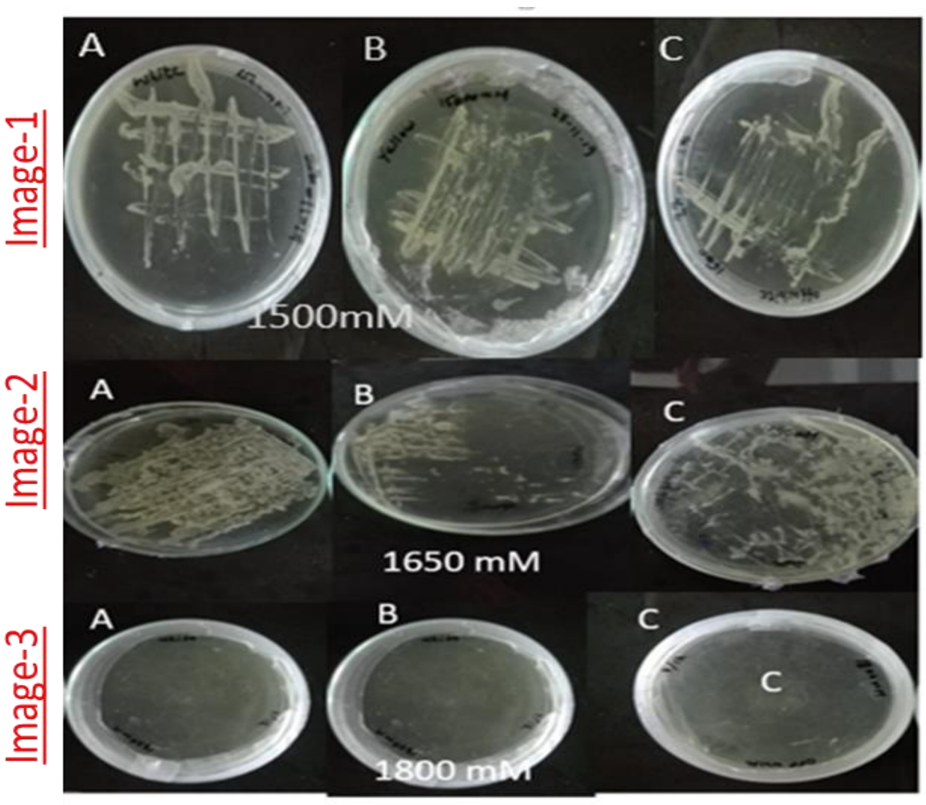

Figure 4: Growth of endophytic bacteria at 2000mM Nacl stress

Image 1: Show 1500 mM Nacl Stress, Image 2: Show 1650 mM Nacl Stress.

Image 3: Show 1800 mM Nacl Stress.

3.2 Selected endophytic bacteria's were positive for catalase test.

To differentiate staphylococci (positive) from streptococci (negative) by using H2O2 solution onto the microscopic slides containing the culture of the isolates separately. Observations were taken for immediate bubble formation as shown in figure 5.

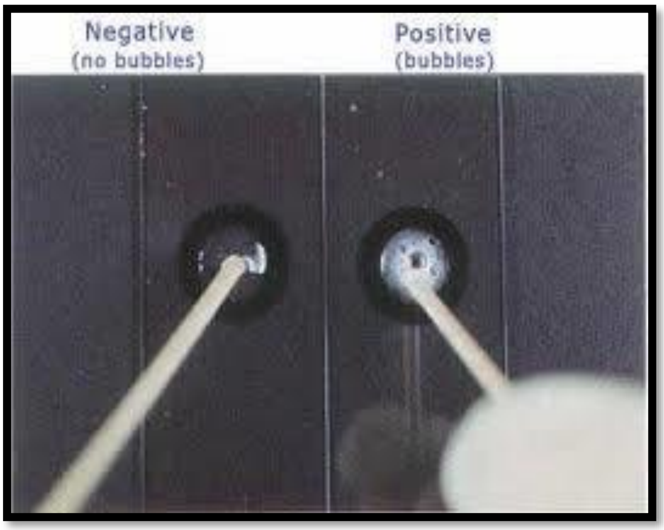

Figure 5: Shows the Release of O2 bubbles which indicated positive catalyse activity

3.3 Endophytic bacteria were slightly positive for ammonia production

3.3.1 Nessler reagent

Is an aqueous solution of potassium iodide (7.0gm) mercuric chloride (10.0gm) and sodium hydroxide .Which is used for the determination of ammonia and specific material and this reagent detect ammonia in a particular substance and the color of solution is change yellow. First Nessler reagent is form and then poured and six falcon tube each with (5 micro liter)then colonies of Endophytic bacteria were picked by using tooth pick and drop and each of six falcon tube after that we kept these falcon tubes in continuous in 37C degree for the 12 hours and the next day the solution of color was changed which shows positive results.

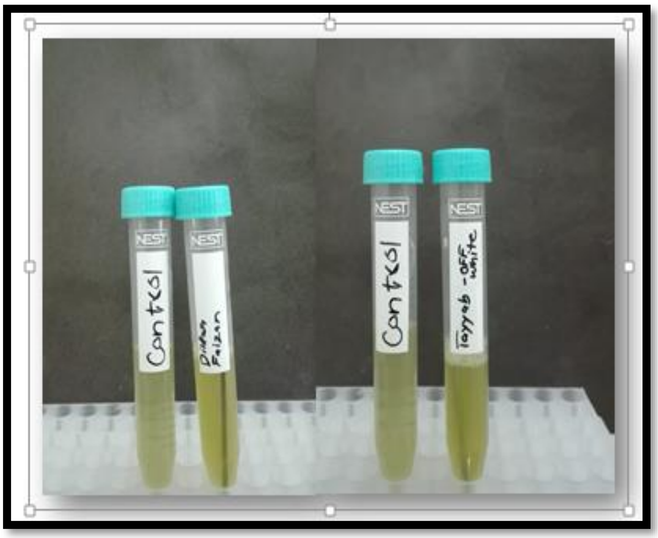

Figure 6: Shows that the solution of colour was changed which shows positive results

4. Discussion

A specific amount of endophytic bacteria were isolated first and then grown on the agar media as it indicates of endophytic isolates. The diversity and beneficial characteristics of endophytic microorganisms have been studied in Plectranthus tenuiflorus medicinal plant. Endophyte as microorganism resist inside the plant without any regards to specific tissues, interactions between plants and bacteria help plants to settle in ecosystem restoration processes (Glick et al., 1995). These interactions may increase the ability of plants to utilize nutrients from the soil by increasing root development, nitrate uptake or solubilizing phosphorus, and to control soil-borne pathogens. Recently, many known as well as new endophytic bioactive metabolites, possessing a wide variety of biological activities as antibiotic, antiviral, anticancer, anti-inflammatory, antioxidant, etc., have been identified .Therefore, it is a huge potential to screen novel, highly active, and low toxicity antimicrobial substances from endophytes (Strobel and Daisy 2003).

Lately there were some reports shown that endophytic isolates also behaves as bio-medicine. Characterization of endophytic bacteria was done on the bases of colony morphology, colony color, cell shape, motility, growth rate, and Gram reaction. The interaction of endophytic bacteria or isolates with microorganism contains nutrients and growth-influencing substances. 51% of biologically active substances are isolated from the endophytic fungi (Strobel, 2003).

Endophytic bacteria are non-pathogenic microorganism that lives or are present in the internal tissues of plant. In most of the research the characterization of the endophytic bacteria is done on the bases of the shape, size and color. As mention in this research the tests that were done on the endophytic bacteria some were positive and some have different results then our research. It also plays an important role in metabolism and physiology of plant.

By using media like nutrient agar media the endophytic bacteria are grown and on the basis of the morphological and biochemical characterization of the endophytes. Root and leaf tissue were selected and it was found that the population density and type of the Endophyte was more in root than in leaves. Many of the bacterial genera encountered in this work were previously reported by Lopez-Lopez *et al.*, and many species of genera *Bacillus* were found by Walker *et al.* in bean seeds. Endophytic population according to cultivar or clone has differences, some specific endophytic bacteria were only isolated from a single studied cultivar.

Endophytic bacteria are bacteria that live in plant tissues without doing substantive harm or gaining benefit other than residency. Both gram-positive and gram-negative bacterial endophytes have been isolated from several tissue types in numerous plant species. These variations are attributed to plant source, plant age, tissue type, time of sampling, and environment. Generally, bacterial populations are larger in roots and decrease in the stems and leaves. All of this research has done for the determination of the prevalence, properties, persistence, and types of endophytic bacteria in agronomic and native plants.

Modification of plants to obtain organisms with improved genetic capabilities and tolerance to different environmental conditions is generally carried out by plant breeding and by integrating foreign DNA into plant genomes to produce transgenic plants. The successful colonization of several crops with such microbes suggests that they can be utilized in future applications, such as delivery of degradative enzymes for controlling certain plant diseases or other useful products.

In our research we have taken samples of endophytic bacteria which were specifically taken from the maize plant and then grown in vitro in agar nutrient media, after the growth different stress tolerant tests were performed to check how much endophytic bacteria from maize have tolerance resistance (salt stress, nessler reagent test and others) with the help of these tests we examine different shape, size and colored bacteria and also the stress level ranks from 50mM to 1650mM.

5. Conclusion

Plants are drastically damaged by changing their environment showing a low yield and growth. But it is known that plant stress tolerance is linked with the associated microbes in plants are ecofriendly approach to better the yield and growth such microbes to be found as rhizosphere and small portion are endophytic in them rhizosphere bacteria are influenced due to environmental factor which cause the low yield and growth efficiency in stress environment,where as endophytes are beneficial over rhizosphere protected from stress conditions. However endophytes are potential tools for the improving of plant growth in high yield and exploring the diversity of endophytes in many stages of plants and their role in growth endophytic bacteria associated with halophytic bacteria are capable to tolerate high salt stress conditions.

Recommendation

1. The isolates maybe used as bio- fertilizers in areas where P and K are found in fixed form or availability of P and K is limited.

2. The isolated Bacteria in this study will be investigated further in fields and greenhouse.

3. The K and P deficient soil condition may improve through isolates.

4. Further investigation needs for complete spectrum of phytohormones and secondary metabolites production.

5. These isolates may be checked for heavy metal resistance and also checked against plant pathogenic strain as a bio-pesticides.

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