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Effect of Nickel and Chromium Metals on the Development of Rhizobial Colonies in *Pisum Sativum* Rhizosphere

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ABSTRACT

The focus of this research is to characterize Rhizobium colonies isolated from root nodules of Pisum sativum plants and determine their tolerance to heavy metals Nickel and Chromium using CFU counts. The results showed that a bacterial colony isolated from root nodules of pea on YEMA medium is a milky white colony with a spherical convex surface. Biochemical analyses of the isolated colonies revealed that they were Rhizobia species, with positive results for oxidase, catalase, and bromothymol blue, but negative results for starch hydrolysis, lipase test, lysine decarboxylase, and caseinase. The presence of high levels of Nickel and Chromium resulted in a decrease in the rhizobial colony. The presence of high levels of Nickel and Chromium resulted in a decrease in the rhizobial colony. All of the colonies thrived well at pH 6 and 7, temperatures of 28º C to 30º C, and a salt concentration of 1%, according to the study.

KEY WORDS: ***Rhizobium, Heavy metal, CFU count, symbiotic relationship, Yeast extract mannitol agar.***

INTRODUCTION

Due to concerns about the human food chain and environmental protection, heavy metal pollution of soils and crops has become a major issue around the world. Metal pollution of soils is common as a result of human, agricultural, and industrial activities (Beladi et al. 2011). Metal remains concentrate in agricultural soils as a result of these operations, posing a risk to food safety and public health (Dary et al. 2010). Because the composition of microbial flora and microbial activity are substantially impacted by metal buildup, soil fertility is lost (Krujatz et al. 2012). These activities result in the accumulation of traces of metals in agricultural soils which pose a threat for food safety and public health (Dary et al. 2010). Some metals, though required in small amounts for organisms, can be hazardous in large amounts. One of the most serious risks to the environment and living organisms is heavy metal contamination (Wo-Niak and Basiak 2003).

In rhizobacteria, Ni is one of the micronutrients required for the production of hydrogenase, which catalyses the oxidation of hydrogen generated by nitrogenase during the reduction of dinitrogen. (Hoffman et al., 2014). Nickelin (HypB), an accessory protein that carries a dual role in Ni mobilisation into hydrogenase and Ni storage in rhizobia, is responsible for Ni supply (Higgins, 2019).

Previous research has shown that metals have a negative impact on microorganism growth, morphology, and activity, including symbiotic nitrogen fixation (Abd-Alla et al. 2014). This symbiosis has been proposed as a way to remove or fix heavy metals from contaminated soils while also improving soil fertility (Dary et al. 2010).

As a result, finding and isolating rhizobial isolates with high levels of heavy metal resistance has been a top priority (Abd-Alla et al. 2012) In metal-contaminated soil, a symbiotic relationship promotes soil fertility while simultaneously extracting or stabilising metals, but has a negative influence on rhizobium colonies (Carrasco et al. 2005). Pollutants in the environment, such as chromium compounds, can negatively affect rhizobia, legumes, and their symbiotic relationships. Chromium toxicity to nodules includes the development of oxidative stress and protein modification (Stambulska et al. 2017). Cr treatment can cause nitrogenase deactivation and reduce nodule performance since nitrogenase is particularly sensitive to oxidation. (Stambulska et al. 2017)

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Pea (*Pisum sativum* L.) is a widely farmed grain legume and fodder crop in peninsular India, and often a pulse crop. Its importance stems from its capacity to form a symbiotic connection with nitrogen-fixing rhizobacteria in the soil, which promotes plant growth and productivity. The goal of this study is to look at how Ni and Cr stress tolerance affects rhizobia's relationship with plants.

METHODOLOGY

SITE SELECTION AND SOIL SAMPLING PROCEDURES

Composite soil samples were collected from crop research centre, Pantnagar (Uttarakhand). At a depth of 0–20 cm, soil was removed from the plough layer. The host plant was not present in any of the soil samples when they were taken. The collected sample was air dried, then crushed with a hardwood roller on a hard wooden slab, sieved at 2mm, and kept in labelled polythene bags. Soil samples were gathered in polythene bags and delivered to 21 pots where pea seeds were planted. *Pisum sativum,* variety Azad seed were brought from Vegetable research centre,Pantnagar and sown in control, treatment and replicate pots.

Under natural settings, all experimental pots were filled with 5 kg of soil and placed in a completely randomised design with twelve replicates. The experiment had six different treatments (each with three triplicates, i.e. six treatments, 12 replicates, and three controls). The plants were taken after 9 weeks of growth. Heavy metals such potassium dichromate ($K_2Cr_2O_7$) and nickel sulphate ($NiSO_4$) were added at concentrations of 10ppm, 50ppm, and 100ppm solution after two weeks. Watering took place twice a week. From December to March, they were allowed to grow for four months.

PHYSICOCHEMICAL ANALYSIS OF SOIL

The pH and EC of the soil were measured in the lab with an Orion pH/EC metre model Li -120 from Elico India Ltd., New Delhi. The Walkley and black method was used to determine the organic carbon content. The alkaline potassium permanganate test was used to estimate available nitrogen in soil.

COLLECTION, ISOLATION, CHARACTERIZATION AND CFU COUNT OF RHIZOBIAL COLONIES.

After 55-60 days, nodules appeared in Pea. Cultivated pea plants were used to collect nodules. Treatment, duplicate, and control were chosen from the several pots. Because nodules can be found on lateral roots and the tap root, plants were uprooted with a knife to avoid severing secondary roots. The nodules were carefully removed using a blade with lateral roots and placed in silica gel vials before being wrapped with cotton wool. (Woomer et al. 2011). The Yeast Extract Mannitol Media (YEMA) is the growth medium used to isolate rhizobia (Vincent, 1970). The following is the YEMA media composition: 10.0 g Mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 1.0 g Yeast Extract, 15.0 g of agar and 1 litre of distilled water. Adjustment was done on the pH of the media to 6.8 using 0.1N NaOH. Dyes were incorporated in the Media: Congo Red (CR) and Bromothymol blue (BTB) that were added at a concentration of 25 ppm.

Surface sterilization of rhizobia from nodules was accomplished by immersing them in 95 percent ethanol for 5-10 seconds, then transferring them to a 2.5-3 percent (v/v) sodium hypochlorite solution and soaking them for 3- 4 minutes. Using clean forceps, the nodules were rinsed in five changes of sterile water. Following surface sterilization, the nodule was crushed in a petri-dish with a pair of blunt-tipped forceps in a huge drop of sterile water. A drop of suspension was streaked onto the agar surface to gradually dilute the suspension. The plates were then incubated in the incubator at 25-30°C. After growth, a single colony was re-streaked again on YEMA culture media for purification (Somasegaran and Hoben, 1985). The appearance, growth rate, and production of alkalinity or acidity were all used to identify typical rhizobia. To differentiate rhizobia from other bacteria, Congo red was added to the media. The viable plate account (CFU/ml) was determined after enumerating rhizobial colonies using the Most Probable Number Method (Vincent, 1970).

GRAM STAINING OF THE BACTERIAL STRAIN

For a more specific identification of rhizobial colonies, pure cultures of bacterial colonies were subjected to gram staining. In a laminar air flow chamber, gram staining was done. The slides were first cleaned in ethanol, and colonies were then marked on them using an inoculating needle and heat fixed. The smears were therefore stained using the techniques below. a) On each slide, apply crystal violet and leave for 1 minute. c) Wash with distilled water c) Iodine as a mordant (1 minute), followed by a 95 percent alcohol wash (30 seconds), and finally distilled water. d) Safranin was added to the slides, which were then rinsed in distilled water and air dried. The entire gram staining technique was done following the Christian Gram technique and Collee JG, Miles RS Mackie (1989).

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EFFECT OF SALT, PH AND TEMPERATURE ON RHIZOBIAL COLONIES

The salinity tolerance of the bacteria was tested by cultivating them on YEMA medium with varied salt concentrations of 1%, 2%, 3%, and 4% (w/v) NaCl. The ability of Rhizobial isolate to grow at different pH levels was examined on YEMA medium using NaOH and HCl to alter the pH to 5.0, 6.0, 7.0, 8.0, and 9.0. Temperature tolerance was tested by growing bacterial cultures in YEMA medium at various temperatures, including 5° C, 28° C, 35° C, and 40° C.

BIOCHEMICAL CHARACTERIZATION OF RHIZOBIAL COLONIES

Catalase Test

This test was conducted and see whether Rhizobium spp. had the enzyme catalase, which hydrolyzes H_2O_2 into H_2O and O_2 in bacterial strains. After making a smear of strain on a clean and dry glass slide, a few drops of H_2O_2 were applied to the slide.

Citrate Utilization Test

In YEM agar medium, sodium citrate and Bromothymol blue (25 mg/l) were used instead of mannitol to examine citrate uptake as a carbon source. Rhizobium spp. isolates were streaked in YEMA medium plates with sodium citrate applied and bromothymol blue as an indicator. Following that, the plates were incubated for 24 to 48 hours.

Bromothymol Blue Test

It distinguishes between fast-growing Rhizobium isolates and slow-growing Rhizobium isolates. To examine if bacteria would react acidically or alkalinely, bacteria were inoculated on YEM agar medium with 0.025 percent bromothymol blue and incubated for 3 to 10 days. On BTB-containing YEMA medium, this test sample was allowed to develop. The positive sample turned yellow after 48 hours of incubation at 280°C due to acid production.

Lipase test

Lipase presence around bacterial colonies was detected by supplementing YEM with 1% (w/v) Tween 80.

Starch Hydrolysis

This experiment was carried out to see if Rhizobium might utilise starch as a carbon source. To assess the ability of bacteria to utilise starch, Starch Agar Medium was inoculated with Rhizobium and then iodine was added. A drop of iodine (0.1N) was dropped into a 24-hour-old culture, which revealed a definite zone of inhibition around the bacterial colonies.

Lysine Decarboxylase Test

Rhizobium strains were streaked on Bromocresol Purple Falkow medium in this experiment (peptone 5 g, yeast extract 3 g, glucose 1g, Bromocresol purple 0.02 g, distilled water 1 liter). The Rhizobial strains were then streaked on the media and incubated for 24 hours at 340°C.

Statistical Analysis

Statistical analysis was done in triplicates for each treatment. The mean and standard error (SE) were calculated using MS Excel, 2007

RESULT AND DISCUSSION

The soil parameters were examined at the commencement of the experiment. All of the treatment plants were given heavy metal solutions. Potassium dichromate solution at concentrations of 10ppm, 50ppm, and 100ppm was applied to three treatments and their replicates. Nickel sulphate solution with concentrations of 10ppm, 50ppm, and 100ppm was applied to three treatments and their replication plants.

Physico-Chemical Analysis of Soil.

The pH is 5.70 on average, which is in the moderate acid range, and the soil is light brown in hue. It has a moderate total nitrogen concentration (0.273%) and an electric conductivity of 25.07 S cm^{-1}. The organic carbon content of soil is 0.70 percent and the organic matter content is 1.204 percent. The heavy metal content of soil in unpolluted soils was within normal ranges.

Morphological characteristics

The isolates were round in shape with an entire edge, and their occurrence on YEMA medium was milky to watery translucent, according to morphological features. Colonies were 2.5 mm in diameter, transparent, and pale pink in colour. Rhizobium was Gram (-ve), motile, rod-shaped, and fast-growing in YEMA medium due to convex elevation.

The confirmation of isolates was done using microscopic observations, and the Rhizobium spp. was determined to be gram negative. The colonies were discovered to be gram negative, with a whitish pinkish tint and rod-shaped bacteria under the microscope.

Characterization of Biochemical Processes

Different biochemical tests were used to characterize the biochemistry of chosen isolates. Oxidase, Catalase, Citrate Utilization, Bromothymol Blue, Lysine Decarboxylase, Lipase, and Caesinase were among the test performed. The isolates in this

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investigation tested positive for oxidase and catalase but negative for lipase, urease, and caseinase.

Isolates were identified as Rhizobium sp. based on biochemical data. The caseinase test in Rhizobium leguminosarum strain is negative, according to Datta et al., 2015. The oxidase test was positive in our findings, with the colonies turning dark purple to black in colour within 5 minutes in the test isolates. The catalase test was found to be positive in this investigation due to the development of bubbles around bacterial colonies. Bubble formation around bacterial colonies of all four strains was also observed by Datta et al. 2015. Javed and Asghari et al. (2008) used the same biochemical procedures to define Rhizobium from root nodules.

The starch hydrolysis assay, that is used to detect the generation of reducing sugar from starch in bacteria, revealed no defined zone around the isolates. Bromocresol purple falkow media was used for the lysine decarboxylase test, and there was no colour change in the Rhizobium-inoculated medium. Citrate was used as a carbon source in this investigation, however there was no colour change, indicating a negative citrate test. Bradyrhizobium japonicum and Rhizobium leguminosarum show no colour change and have a negative citrate test, according to Datta et al. 2015. The existence of Rhizobium spp. in our study is confirmed by all of the above observations and outcomes.

pH, NaCl, and temperature tolerance in rhizobial colonies exposed to varied Nickel and Chromium concentrations.

The ideal temperature for root nodulating bacteria growth ranged from 25°C to 30°C in this study. The optimal temperature for Rhizobia growth was determined to be 28°C, with moderate growth seen at 35°C. The optimal pH for rhizobial growth was found to be between 6 and 8 in this study. At pH 8 and pH 9, isolates grew at a very slow rate. In this study, it was discovered that Rhizobia grew at varied rates at different concentrations of NaCl, with the maximum growth rate occurring at 1% (w/v) NaCl and the lowest at 4% (w/v) NaCl. However, as the salt concentration was increased, the percentage of tolerant isolates declined, and only 11.1 percent of the isolates tolerated 5 percent NaCl.

Effect of Nickel and Chromium on the *Pisum sativum* rhizosphere and rhizospheric bacterial colonies

Total CFU count of bacterial isolates in the presence of Nickel

Pisum sativum plants in the control variant (c) were well established two months following germination, but had a lower number of root nodules. In the soil, the CFU (colonies forming units) ml-1 of free-leaving bacteria was 8 x 106 CFU ml-1, with an aspect of The Most Probable Number Method (Vincent, 1970) was used to count soil bacteria and determine the number of viable plate count (CFU/ml). Mucous production and colony shape were among the morphological features studied. During the growth of the isolates, the pH of the medium changes (Somasegaran and Hoben, 1985).

The number of bacteria in the Ni-treated variations was lower than in the control (C), with 2.77 x 10^6 CFU ml^{-1}in N1 (26.3 mg Ni kg^{-1}soil), 2.60 x 10^6 CFU ml^{-1}in N2 (131.5 mg Ni kg^{-1}soil), and 2.00 x 10^6 CFU ml^{-1}in N3 (263.0 mg Ni kg^{-1}soil). The plants in N1 were well-developed, with large nodules on the roots. The plants in N2 were likewise highly developed, having a large number of nodules on the roots, although not as many as in N1. The plants in N3 were tiny and had no obvious nodules on the roots.

Total CFU count of bacterial isolates in the presence of Chromium

Plants cultivated in C1 (56 mg Cr kg^{-1} soil) were smaller than those produced in N1. Plants and nodules were small, but noticeable on the roots; some were lobated, and they were usually bundled in bundles. The CFU ml^{-1} value was 3.53 x 10^6, which was lower than the control variant. The plants in the C2 (280 mg Cr kg^{-1} soil) and C3 (570 mg Cr kg^{-1} soil) variants were tiny and lacked nodules, suggesting that the amount of Cr employed for treatment was hazardous. According to Smith (1997), the absence of nodulation was indicative of either a very low rhizobial population, below the detection limit of the plant infection assay, or a very low rhizobial population, below the detection limit of the plant infection assay or the complete complete absence of rhizobia from soil.

CONCLUSION

The proposed research looked at the effects of nickel and chromium on the establishment of rhizobial colonies in the root zone of Pisum sativum, as well as counting them using colony forming units (CFU). The study found that the heavy metals Ni and Cr impede the formation of Rhizobial colonies. Rhizobium is a gram-negative bacterium that forms a symbiotic relationship with leguminous plant roots. For biological nitrogen fixation, screening and selecting rhizobial colonies from the rhizosphere is critical. The milky white colony with round convex surface was isolated from bacterial colonies from pea root nodules that could not absorb red colour on YEMA medium. Many biochemical tests of the isolated colonies were positive, such as oxidase, catalase, and bromothymol blue, while starch

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hydrolysis, lipase test, lysine decarboxylase, and caseinase were negative, indicating that the colonies recovered from pea plants are Rhizobia species. All of the colonies thrived well at pH 6 and 7, temperatures of 28°C to 30°C, and a salt concentration of 1%, according to the study.

The current study appears to be a promising approach for determining the best strategy for isolating a rhizobial colony from a pea plant that might be employed in nitrogen fixation, but more research should be done using genetic engineering and biotechnology techniques.

Table 1. Morphological study of *Rhizobial* colony

S.No	Colony characteristics	*Rhizobial* colonies
1.	Shape	Circular
2.	Size	2.5mm
3.	Color	White
4.	Opacity	Transparent
5.	Bacterium shape	Rod shaped
6.	Gram nature	Gram negative
7.	Motility	Mobile

Table 2. Various biochemical tests for Rhizobial colonies

Tests	Control	N_1	N_2	N_3	C_1	C_2	C_3
Bromothymol blue	+	+	+	+	+	+	+
Caesinase	–	–	–	–	–	–	–
Catalase	+	+	+	+	+	+	+
Citrate	–	–	–	–	-	-	-
Lipase	–	–	–	–	–	–	–
Lysine decarboxylase	–	–	–	–	–	–	–
Oxidase	+	+	+	+	+	+	+
Starch hydrolysis	–	–	–	–	–	–	–

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Table 3. pH, NaCl, and temperature tolerance in rhizobial colonies exposed to varied Nickel and Chromium concentrations.

Rhizobium colonies	pH		NaCl			Temperature (^{0}C)			
	4 pH	9 pH	0.1	0.3	0.5	10	20	30	40
C	+	-	+	+	+	-	+	+	+
N_1	+	-	+	+	+	-	+	+	-
N_2	+	-	+	+	+	-	+	+	-
N_3	-	-	+	+	+	-	+	+	-
C_1	+	-	+	+	+	-	+	+	-
C_2	+	-	+	+	+	-	+	+	-
C_3	-	-	+	+	+	-	+	+	-

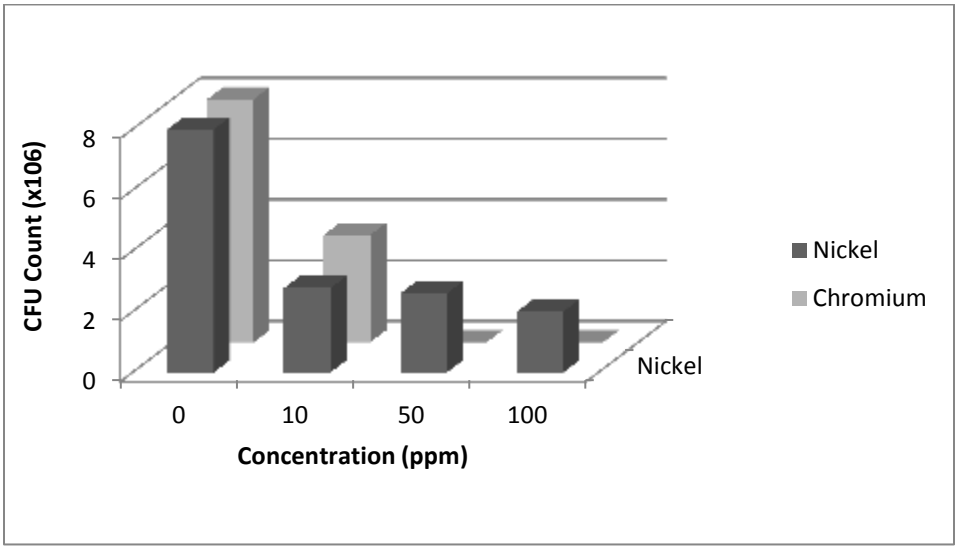

Figure1 .Comparison chart of rhizobial colonies in presence of Ni and Cr

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