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Study of morphological and Biochemical characterization of *Rhizobium* isolated from Root nodules of *Cajanus cajan* L. exposed to Cement dust pollution

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Abstract

In the present study, 11 rhizobium strains were isolated from root nodules of redgram (*Cajanus cajan)* crop which is exposed to cement dust of Kalaburagi cement industry of sedam taluk and Chettinad cement industry of Chincholi taluk and control field is far away from these two cement industries. Rhizobium isolates were isolated using selective media such as Yeast Extract Mannitol Agar media (YEMA). The isolates were identified as rhizobium on the basis of their morphological, biochemical and confirmatory tests. All isolates were gram-negative, rod shaped and poor absorption of red color when cultured in YEMA containing Congo red. In the ketolactose test yellowish zone of Cu_2O not found. All isolates showed fast growth in YEMA+BTB. Based on the Morphocultural and Biochemical studies, results were confirmed that the isolated strains were rhizobium.

Key words : Cement dust**,** Soil, Redgram crop, Rhizobium, Root nodules.

Soil is a natural resource, which nurture the plants and it is natural habitat for microorganisms. Now a day's soil is becoming polluted due to various industrial activities and anthropogenic activities such as increasing human population, industrialization, deforestation and urbanization[26]. Cement industries causes various environmental problems in the surrounding areas which spreads dust over large areas through wind[2,36]. Cement dust is released from various processes by handling, spillages, leakages, quarrying of limestone and packing and transporting of cement from the factory[1]. Industrial pollution causes changes in the physico- chemical and biological properties of water, air and soil that will be negative effect on plants and living beings[20]. The pH of cement polluted soil was alkaline in

nature. Similar studies reported result of increased levels of soil pH due to cement dust pollution [17]. Cement dust pollutants closure the leaf stomata, decline photosynthetic ability and reduction in growth and crop yield is reported[8].

Cement dust decreased the crop yield and concentration of chlorophyll in various crops is also reported[25]. In agricultural ecosystem rhizobium bacteria plays a big role in biological symbiotic nitrogen fixation with leguminous[21]. The main objective of this study is isolation and identification of *Rhizobium* bacteria from root nodules of red gram cultivated fields, around Kalaburagi cement industry of Sedam taluk and Chettinad cement industry of Chincholi taluk of Kalaburagi district.

Collection of root nodule samples from selected agriculture fields :

Root nodules of redgram crop were collected from three different study sites namely Kalaburagi cement industry Sedam taluk, Chettinad cement industry Chincholi taluk and control field is far away from these two cement industries, which has same distance from these two cement industries. From redgram fields roots were uplifted carefully and placed in clean polythene bag and labeled and brought to the laboratory for further studies[38].

Isolation of Rhizobium from root nodules:

Nodules were separated from the collected roots. Healthy and unbroken root nodules were selected for the isolation of rhizobium by using standard protocol[30]. The selected root nodules were first washed with running tap water to remove soil particles and nodules surface sterilized with 1% sodium hypochlorite solution for 3 min, soon after nodules were washed 7 changes of sterilized distilled water. Surface sterilized nodules were crushed in a test tube with sterile glass rod to get a milky suspension[19]. A loop-full of suspension was streaked on Yeast Extract Mannitol Agar (YEMA) media containing Congo red (0.00125 mg/kg) and incubated at 28^{o}C for 3-5 days[28, 37]. After incubation single colonies were selected and re-streaked on YEMA plates for obtaining pure cultures.

Morphological characterization :

Morphological characteristics of all the isolates were determined by selected parameters such as colony- shape, size, color, texture and elevation. After an incubation of 2 to 3 days at 28^{o}C on YEMA plate[37]. Pure and fresh cultures of isolates were used for Gram staining technique[3]. This culture were smeared on microscope slide and air dried, heat fixed and prepared slide were observed under 40x and then under oil immersion on a compound microscope. Motility test of all the isolates of *Rhizobium* was done by Hanging drop method[5].

Confirmatory tests of Rhizobium :

Confirmatory tests were performed to confirm the isolated organisms as Rhizobia from other Agrobacterium.

Growth on YEMA with Congo red :

YEMA medium with Congo red (2.5 ml of 1% solution) was prepared. Bacterial

isolates were streaked on this medium and incubated at 28°C for 3-5 days. Little or no absorption of Congo red was confirmatory of *Rhizobium* whereas Agrobacteria, will absorb Congo red dye strongly[9,37].

Growth on Glucose Peptone Agar (GPA) medium :

Glucose peptone agar media was prepared by adding Bromocresol purple indicator. In this medium bacterial isolates were cultured and incubated at 28°C for seven days. Poor growth or no growth in this medium was confirmatory test for *Rhizobium*[37].

Keto-lactose Test :

Keto-lactose medium was prepared and bacterial isolates were streaked on this medium and incubated at 28°C for a week. After the incubation, Plates were flooded with Benedict's reagent, and kept for 1 hour in an incubator and then results were observed, color changes from blue to yellow around the colonies is indicates conversion of lactose to lactonic acid and bacterial isolates showing negative reaction for ketolactose test were considered positive for *Rhizobium*[4,13].

Bromothymol blue (BTB) test :

Yeast Extract Mannitol Agar (YEMA) medium containing bromothymol blue (0.00125 mg/kg) was used to identify fast and slow growing rhizobium isolates. All isolates were streaked on YEMA+BTB media. Fast growers produce acidic reaction by changing color of the media from green to yellow after incubation for 48 hours at 28°C and slow growers produce alkaline reaction by changing color of the media green to blue[29].

Hoffer's Alkaline broth Test :

This test is performed to know that *Agrobacterium* grows at higher pH level whereas *Rhizobium* will not grow. Hoffer's Alkaline broth was prepared by increasing pH 11.0 by adding NAOH. Bacterial isolates was inoculated in this broth and incubated at 28°C for seven days. Incapability of the isolates growth on YEM broth confirmatory test for *Rhizobium*[12].

Biochemical Characterization of Rhizobium:

Catalase test :

This test was performed to study the presence of Catalase enzyme. Catalase activity of *Rhizobium* was tested by using 24h colonies on a glass slide, by adding one drop of 30% H_2O_2. Appearance of gas bubbles indicated the presence of Catalase enzyme[15].

Methyl red test :

Rhizobium isolates were inoculated in methyl red-Voges Proskauer broth of about 5 ml/test tube and incubated at 30°C for 48 hours, after incubation 5 drops of methyl red indicator was added to each test tube. Red color broth indicates positive result while yellow color indicates a negative result[7].

Voges-Proskauer test :

Rhizobium isolates were inoculated in methyl red-Voges Proskauer broth of about 5 ml/test tube and incubated at 30°C for 48

hours. After incubation, 12 drops of V-P reagent I and 2-3 drops of V-P reagent II were added. The development of a red color is indicates positive result and no change in color is a negative result[16].

Starch hydrolysis test :

Starch hydrolysis test was performed to examine the ability of *Rhizobium* to use starch as carbon source[6]. *Rhizobium* isolates were inoculated on starch agar medium by streaking once across the surface and incubated at 30°C for five days. After incubation 5 mL of iodine solution (0.340 g iodine and 0.660 g potassium iodide in 100 ml distilled water) was added. Appearance of a clear zone around colonies indicates positive result[11].

Indole test :

Tryptone broth was prepared and poured into 10 ml test tubes then autoclaved (121°C; 15 psi; 15 min). *Rhizobium* was inoculated in broth and incubated at 30°C for two days. Uninoculated broth was kept as control. After incubation period, 1 ml of Kovac's reagent was added to each test tube. Tubes were shaken 10-15 min and allowed to stand some more time to get reagent surface of the tube. The formation of a deep red color in the top layer of the tube is indicated a positive result and absence of red color indicated a negative result[11,15].

Citrate utilization test :

The citrate utilization test was determined by replacing mannitol in YEM agar with an equal amount of sodium citrate and bromothymol blue (25 mg/l). Media was prepared and all isolates were inoculated by streaking on media and incubated 30°C for 48 hours[14]. After incubation, a positive result showed color changed from green to blue.

Morphological characteristics :

All the isolates were identified on the basis of morphological and microscopic features. Most of the isolates were found creamy white colony with round shape, butyrous texture characteristics. All isolates were found to be Gram negative and rod shaped. Most isolates were motile cells. The complete result is represented in Table-1.

Confirmatory tests :

Several confirmatory tests were performed to confirm the isolated organisms as Rhizobia. All confirmatory test results of bacterial isolates were represented in Table-2.

Organisms were inoculated on YEMA medium with Congo red (Congo red Test) (2.5 ml of 1% solution), little or no absorption of Congo red was observed. This confirms that the isolated organisms were *Rhizobium.*

The organisms grown on Glucose peptone agar (GPA Test) media by adding Bromocresol purple indicator. Poor growth or no growth in this medium was confirmed that the isolated organisms were *Rhizobium.*

Similarly when the organisms grown on Keto-lactose (KLA Test) medium, flooded with Benedict's reagent, no yellow colour formation was observed around the colony, this conirmed that the isolated organisms were *Rhizobium.*

Further Bromothymol blue (BTB) test was done using Yeast Mannitol Agar (YEM) with 1% bromothymol blue media to identify fast and slow growing rhizobium isolates. Fast growers produce acidic reaction by changing color of the media from green to yellow after incubation for 48 hours at 28°C and slow growers produce alkaline reaction by changing color of the media green to blue.

Hoffer's Alkaline broth Test were performed to know the growth of *Rhizobium,* usually *Rhizobium* will not grow at higher pH whereas Agrobacterium grows at higher pH level. Due to the high pH in the growth medium, the rhizobium showed poor or no growth.

Biochemical characteristics of isolates :

Selective biochemical tests were performed for all isolates. No bubbles formation was formed for catalase test. All isolates showed negative result for VP test and starch hydrolysis test. For citrate utilization test all isolates were showed negative results except CFRN1, CFRN2, CFRN3, and CFRN4. Except isolate KLRN1 and KLRN2, all isolates were found negative result for methyl red test and Indole test respectively. The results of biochemical tests summarized in Table-3.

The environmental pollution causes reduction in plant growth and decreased photosynthesis[10]. Nodules are formed spherical or cylindrical growth in the roots of leguminous crops. They are formed as a result of an infection by bacteria[32]. They play a prominent role in the process of nitrogen fixation in leguminous crops. In the present study, control field redgram crop has more number of root nodules and cement dust polluted agriculture fields of redgram have less number of root nodules. Due to cement dust, the reduction in

Table-1. Morphological characteristics of isolates

Isolate code	Colony size (mm)	Colony shape	Colony color	Texture	Nature	Cell size and shape	Cell motility	Gram staining
CFRN1	Medium	Round	Cream	Butyrous	Opaque	Short rods	+	-ve
CFRN2	Medium	Round	Cream	Butyrous	Opaque	Short rods	+	-ve
CFRN3	Medium	Round	Cream	Butyrous	Opaque	Short rods	+	-ve
CFRN4	Medium	Round	Cream	Butyrous	Opaque	Short rods	+	-ve
KLRN1	Small	Round	White	Watery	Translucent	Long rods	_	-ve
KLRN2	Small	Round	White	Watery	Translucent	Long rods	+	-ve
KLRN3	Large	Round	Cream	Butyrous	Opaque	Short rods	+	-ve
CNRN1	Large	Round	Cream	Butyrous	Opaque	Short rods	_	-ve
CNRN2	Large	Round	Cream	Butyrous	Opaque	Short rods	+	-ve
CNRN3	Large	Round	Cream	Butyrous	Opaque	Short rods	_	-ve
CNRN4	Large	Round	Cream	Butyrous	Opaque	Short rods	+	-ve

NOTE: CFRN- Control field root nodule; KLRN- Kalaburagi root nodule cement field; CNRN-Chettinad root nodule cement field.

Table-2. Confirmatory tests of isolates

Isolate code	Congo red Test	GPA Test	KLA Test	Acid/Alkali Test	Hoffers Broth Test
CFRN1	+ve	Poor growth	No yellow color	Yellow/Fast	Poor Growth
CFRN2	+ve	Poor growth	No yellow color	Yellow/Fast	Poor Growth
CFRN3	+ve	Poor growth	No yellow color	Yellow/Fast	Poor Growth
CFRN4	+ve	Poor growth	No yellow color	Yellow/Fast	Poor Growth
KLRN1	+ve	Poor growth	No yellow color	Yellow/Fast	Poor growth
KLRN2	+ve	Poor growth	No yellow color	Yellow/Fast	Poor growth
KLRN3	+ve	Poor growth	No yellow color	Yellow/Fast	Poor growth
CNRN1	+ve	Poor growth	No yellow color	Yellow/Fast	No growth
CNRN2	+ve	Poor growth	No yellow color	Yellow/Fast	No growth
CNRN3	+ve	Poor growth	No yellow color	Yellow/Fast	No growth
CNRN4	+ve	Poor growth	No yellow color	Yellow/Fast	No growth

NOTE: CFRN- Control field root nodule; KLRN- Kalaburagi root nodule cement field; CNRN- Chettinad root nodule cement field.

Table-3. Biochemical characteristics of isolates

Isolate code	Citrate utilization test	Catalase test	Methyl red test	VP test	Indole test	Starch hydrolysis test
CFRN1	+	-	-	-	-	-
CFRN2	+	-	-	-	-	-
CFRN3	+	-	-	-	-	-
CFRN4	+	-	-	-	-	-
KLRN1	-	-	+	-	-	-
KLRN2	-	-	-	-	+	-
KLRN3	-	-	-	-	-	-
CNRN1	-	-	-	-	-	-
CNRN2	-	-	-	-	-	-
CNRN3	-	-	-	-	-	-
CNRN4	-	-	-	-	-	-

NOTE: CFRN- Control field root nodule; KLRN- Kalaburagi root nodule cement field; CNRN- Chettinad root nodule cement field.

the number of root nodules were also reported in various crops such as Chickpea[24], Lupine[33], French bean[34] and Common bean. The reduction in number of root nodules may be due to the excessive deposition of cement dust in the soil, due to deposition of dust particles on leaf surface reduce amount of light available for photosynthesis and that changes the soil pH. The increased soil pH has an inhibitory effect on nodule formation[22]. The poor growth of root nodules may be due to poor inhalation of nitrogen fixers in cement dust polluted soil[31]. It may also be happens due to the deposition of cement dust pollutants which may become harmful to the population of *Rhizobium* as suggested by Thangarasu[34].

In the present study, a total of 11 bacterial strains were isolated from root nodules of redgram crop, four isolates from control field and seven isolates from cement polluted field. Isolates were characterized based on their morphological, biochemical and confirmatory tests. The use of Yeast Extract Mannitol Agar (YEMA) medium for the isolation of Nitrogen fixing (Rhizobia) bacteria has been reported by research workers[18,23,27]. For morphological studies all the isolates were found to be round shaped, large, medium and small sized, white and creamy color with translucent and opaque colonies on Yeast Extract Mannitol Agar (YEMA) medium. All isolated Rhizobium strains were gram negative, short rods and motile cells. For confirming the isolates as rhizobia, 5 confirmatory tests were performed. All isolates showed no absorption of Congo red dye on YEMA-Cr media which is consistent with the results of researcher[35] who reported that rhizobia didn't absorb Congo red dye or absorbed very weekly compared with other bacteria. In glucose peptone agar media all the isolates showed poor growth which is similar results of either no growth or poor growth on GPA media[37]. While further confirming all 11 isolates showed poor growth on GPA medium. Result of ketolactose test found all the isolates showed negative result for the production of 3-ketolactose from lactose. All strains of rhizobium have shown negative result for Hoffers alkaline broth test. All the isolates were fast grower and produced acid in Bromothymol blue test. In Congo red all the isolates showed poor absorption of dye Cong red. This gives further evidence for the purity of Rhizobial isolates[30].

Biochemical tests of all the isolates were showed negative results for catalase test, VP test, and starch hydrolysis test. For methyl red test all isolates showed negative result except KLRN1 and for indole test all isolates showed negative result except KLRN2. The isolates CFRN1, CFRN2, CFRN3, and CFRN4 were showed positive result and remaining isolates showed negative result for citrate utilization test. These tests are essential to distinguish *Rhizobium* and *Agrobacterium.*

From the present study it can be concluded that four strains from control field, three strains from kalaburagi cement industry & four strains from chettinad cement industry were isolated from redgram crop. Based on morphological, confirmatory and biochemical characteristics the isolated strains were confirmed as *Rhizobium*. Further studies are necessary to evaluate the potential nitrogen fixing bacteria from the isolated *Rhizobium* strains.

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