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INVESTIGATIVE STUDY OF *AGROBACTERIUM*, A NATURAL GENETIC ENGINEER ISOLATED FROM RHIZOSPHERIC SOIL

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Abstract:

In the present study, *Agrobacterium* was isolated from rhizosphere soil obtained from Botanical Garden of New Arts Commerce and Science College, Ahmednagar by using selective Yeast Mannitol (YEM) medium. On the basis of morphological, biochemical and gram characters of isolates were compared with Bergy's Manual,it was confirmed as *Agrobacterium*. Isolate was further characterized for presence of Ti plasmid. An Alternative Boiling of Miniprep method is used for plasmid isolation was very efficient and gives reproducible isolation of Ti plasmid in short time. Sensitivity to different antibiotics viz: Cefotaxime, Streptomycin and Penicillin was done by disc diffusion method and it showed high sensitivity towards Cefotaxime, moderate sensitive towards Streptomycin, and slight resistant towards Penicillin. Effect of various temperatures (25ºC, 35ºC and 40ºC) on growth of *Agrobacterium* and its Ti plasmid was studied and it was found that high temperature (40ºC) affected growth of *Agrobacterium* but had no effect on Ti plasmid. To our knowledge, it was first attempt to investigate the stability of the Ti plasmid at different temperatures which reveals that Ti plasmid of this isolate is stable even at 40ºC. Further, the Restriction Fragment Length Polymorphism (RFLP) analysis showed no fragment patterns on agarose gel for EcoRI, HindIII and BamHI which clearly indicated that there was no restriction sites for these endonucleases.

Keywords: *Agrobacterium*, Ti-plasmid, Antibiotic sensitivity, Temperature optimization, RFLP analysis

I. Introduction:

Agrobacterium truly a remarkable and fascinating organism spread ubiquitously and widely on this global earth. *Agrobacterium tumefaciens* is soil dwelling, gram negative, non-spore forming, motile, rod shape organism. This study has revolutionized in the field of plant molecular genetics over the last 100 years (Nester, 2014). Since then, it has given birth to a whole new era that are only dedicated to the genetic modification of plants and its study resulted in many surprising observations. Initially, studies were aimed solely at identifying the cause of destructive galls on ornamental plants and fruit trees (Kado *et al*.2010). First in 1907, two American plant pathologists Erwin Smith and Charles Townsend reported that agent causing common destructive disease to variety of ornamental plants and named

it as *Bacterium tumefaciens* Armin Braun (1947) demonstrated the concept of **Tumor inducing principle (TIP)**. During 1969-1971, it was suggested that the TIP was DNA that was transferred from *Agrobacterium* into plant cell. *Agrobacterium* species that are pathogenic to plants, including *A. tumefaciens*, *A. vitis*, *A. rubi*, and *A. rhizogenes*, all carry mega plasmids. By contrast, non-pathogenic strains either lack these plasmids entirely or carry mutant forms of plasmids. A strict requirement of the Ti plasmid for virulence was established through mutational analyses and by a demonstration that the introduction of Ti plasmids into non-pathogenic species that converts these non-pathogenic species into tumor-inducing pathogens (Teyssier - Cuvelle *et al.* 2010). Ti plasmids induce a disease called Crown Gall, which caused formation of undifferentiated plant tumors at the plant crown i.e., near the soil level at junction of root and plant stem. Another root inducing or Ri mega plasmids carried by *A. rhizogenes* instead induce hairy root disease, which form hairy roots (Chawala H.S, 2004). Last many years, we experience beauty of this very small microorganism as Natural Genetic Engineer. The present study aimed to check out existence of *Agrobacterium* in rhizosphere area and to characterize that isolated *Agrobacterium* in detail.

II. Materials and Methods:

Isolation of *Agrobacterium* from soil sample

Rhizospheric soil samples were collected from Botanical Garden of New Arts, Commerce and science college of Ahmednagar. The 0.1gm of soil sample was serially diluted by using 0.85% autoclaved saline solution up to 10^{-8}. The dilutions were spread onto YEM agar plates and incubated at 28ºC - 30°C for 48 hours. After 48 hours, isolates were streak again on YEM plates and gram staining were performed. For further purification isolates were re-streaked on YEM agar plates. Morphological characters like size, shape, color, margin, opacity, consistency, elevation, Gram character of isolated colony were observed and noted. The isolated colonies were characterized biochemically as per Bergey's Manual of Determinative Bacteriology. So IMVIC ,Catalase, Oxidase, Citrate utilization was performed.

Confirmation of Gram-negative isolate

Bacterial colony was mixed with three drops of 3% potassium hydroxide solution and observed viscosity of culture.

Production of 3- Ketolactose

Production of 3- Ketolactose was done by the method described by **Bernaert's and DeLay (1966)**. Lactose medium agar plates were prepared and loopful culture was inoculated on it and incubated these plates in incubator for 48 hours at 28°C to 30^0 C. After 48 hour's plates were flooded with 1 to 5 ml of Benedict's reagent and plates were observed after 2-3 hours.

Growth on Hofer's medium:

Hofer's agar medium plates were prepared for further confirmation of *Agrobacterial* isolates because this medium is selective for *Agrobacterium* species (Hofer, 1935; Gaur and Sen, 1981). The single colony of isolates streak on to the Hofer's agar plates and incubated at 28ºC-30ºC for 48 hours.

Antibiotic sensitivity test:

The isolates were screened for its antibiotic sensitivity. Antibiotic sensitivity assay was performed by disc diffusion method (Bauer-Kirby method 2009). Antibiotics like streptomycin, cefotaxime, penicillin were used to check antibiotic sensitivity.

Isolation of Ti plasmid from isolated *Agrobacterium*

Isolation of Ti plasmid was done as per **Alternative Boiling by Miniprep method** (Holmes and Quigley 1981). Isolated *Agrobacterium* was inoculated in Luria Bertani broth and incubated at 25º C to 30 ºC for 48 hours. Before starting Miniprep, water was boiled and fresh lysozyme STET (8% sucrose, Tris-HCL pH 8, 50 mM EDTA pH 8) mixture was prepared. After 48 hours, 2 ml culture was centrifuged at 5000 rpm for 5 minutes and pellet was collected. Then add 500 µl of STET solution to each vial and vortex it for few seconds to break up the pellet. And again, centrifuged it at 5000 rpm for 5 minutes. After centrifugation, pellet was collected and 350 µl STET solution added into the pellet and resuspend the pellet by gentle vortexing. Add 25 µl lysozyme solutions and mix properly. Immediately place these vials into boiling water bath for 1 to 2 minutes and centrifuge at 14,000 rpm for 10 minutes, transfer the supernatant into new vial without disturbing the pellet add 200µl isopropanol to each vial and centrifuge at 10000 rpm for 5 minutes. Discard the supernatant and wash the pellet with 500µl 70% ethanol. Air dry the pellet for 10 minutes, resuspends each in 100µl of T.E. buffer and then gentle vortex it. Load the sample in well of 1% agarose gel for electrophoresis and bands were observed under UV Trans illuminator.

Bacterial growth analysis at various temperature:

In order to check out growth of *Agrobacterial* isolate at different temperatures, Luria Bertani broth was prepared. Loopful culture was inoculated into broth and incubated these broths at different temperature like 25ºC, 37ºC and 40ºC.

To analyze stability of Ti plasmid at different temperature:

Ti plasmid was isolated from *Agrobacterium* that were incubated at 25ºC, 37ºC, 40ºC and analyzed by agarose gel electrophoresis.

RFLP (Restriction Fragment Length Polymorphism) analysis of isolate:

The plasmid DNA of isolate and Plasmid DNA of standard strain of *Agrobacterium tumefaciens* strain 2146 were isolated by **Alternative Boiling of Miniprep Method.** The vials were placed on ice containing the restriction enzymes. The vials were thawed which containing Enzymes Assay Buffer. Reaction mixtures were prepared by adding 34 µl of Nuclease free water, 10µl of each of standard Ti plasmid and isolate's Ti plasmid ,10 µl of Assay Buffer, 1µl of restriction enzyme Eco R1 into each vial. Similar procedure was also followed by using Restriction Enzymes Hind III and Bgl 1. The vials were mixed by tapping or gentle vortexing and incubated at 37ºC for 1 hour. The 1% gel was prepared followed restriction digestion, 5µl of gel loading buffer was loaded in the wells of agarose gel with 10µl of ready to use low range DNA ladder in one vial as marker. Order was noted down in which the samples and ladder was loaded. Samples were electrophoresed at 50 to 100 volts for 1 to 2 hours and then visualized under UV trans illuminator.

III. Results and Discussion

Isolation of *Agrobacterium*

Agrobacterium was isolated from Rhizosphere soil sample showed visible colonies on Yeast Mannitol Agar (YEM) (Figure 1a) because they used mannitol as their carbon source. Colony was appeared as non-pigmented mucoid and slimy. Gram staining analysis was performed and it showed that isolate is Gram Negative rods shaped (Figure 1b). Further, this Gram-negative character was confirmed by 3% Potassium Hydroxide, there was viscous appearance observed which reconfirmed that the isolate was Gram Negative.

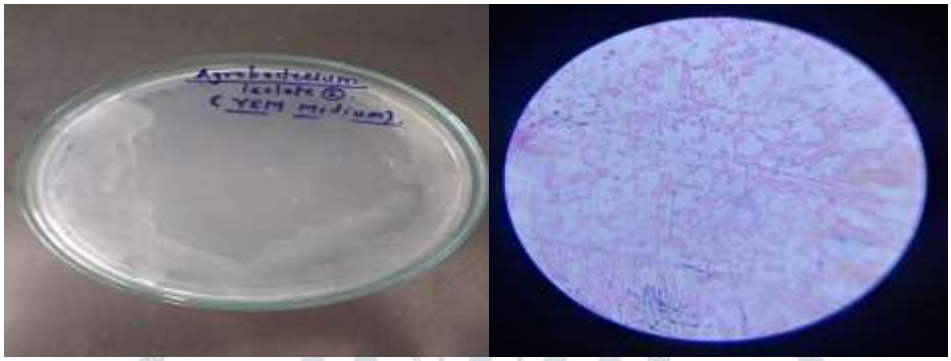

Figure 1: a. Growth of *Agrobacterium* isolates on YEM medium
b. Gram negative rods.

Morphological characteristics of isolate:

The size of the isolated colony was 3 mm, white, circular, and non-pigmented, transparent and mucoid (Table 1). It was convex and had entire margin that is general appearance of *Agrobacterium*.

Sr. No.	Characters	Observation
1.	Size	3 mm
2.	Shape	Circular
3.	Color	White
4.	Margin	Entire
5.	Opacity	Transparent
6.	Elevation	Convex
7.	Consistency	Sticky (mucoid, slimy)
8.	Gram character	Gram Negative

Table 1: Morphological characteristics of isolate

Biochemical characteristic of isolate:

Isolate was further characterized biochemically as per Bergey's Manual of Determinative Bacteriology. As per result, isolate was positive for Catalase, Oxidase, Methyl Red, Citrate utilization and showed negative test for Indole and VP. Biochemical characteristics were summarized as follows:

Sr. No.	Tests	Observations
1.	Catalase	Positive
2.	Oxidase	Positive
3.	Indole test	Negative
4.	Methyl red test	Positive
5.	Voges-Proskauer test	Negative
6.	Citrate utilization test	Positive
7.	3-ketolactose test	Positive

Table 2: Biochemical Characteristics of isolates

Below figures showed the results of Biochemical tests:

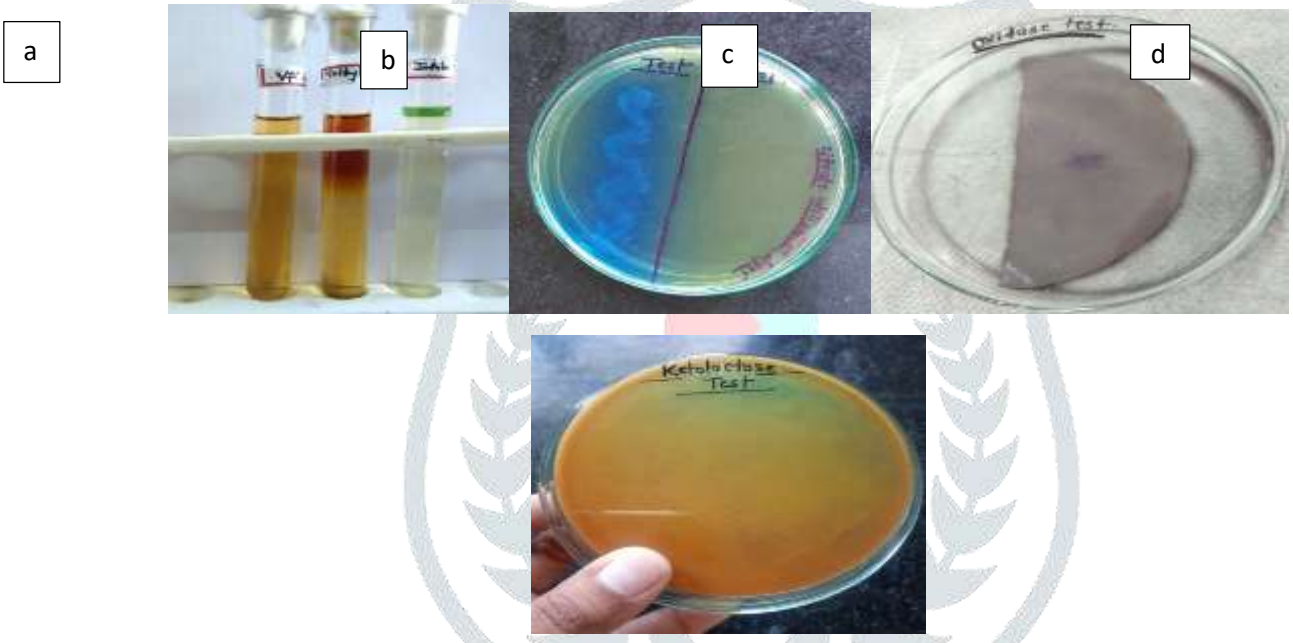

Fig. 2. a. MR-VP test, Methyl Red test and Indole test b. Citrate utilization
c. Oxidase test d. Ketolactose test

Growth on specific Media

Figure 3: Growth of *Agrobacterium* on MacConkey agar and Hofer's medium

From all morphological characters, biochemical tests as per Bergey's Manual, growth on specific medium and test of 3-Ketolactose production, it was identified that the isolated organism as *Agrobacterium*.

Analysis of antibiotics sensitivity of isolate:

The zones of inhibitions were observed on Luria Bertani plates, as shown in Figure 4. The diameter of zones for cefotaxime was 54 mm, 22 mm for streptomycin and 1 mm for penicillin. Therefore, the results showed that isolate was highly sensitive to Cefotaxime, moderately sensitive to streptomycin whereas, it was slightly resistant to penicillin. So, these antibiotics may be used to suppress Agrobacterial infection as *Agrobacterium* is serious pathogen for many economically important plants crops.

Figure 4: Antibiotic sensitivity test against Cefotaxime,Streptomycin,Penicilin

Isolation of Ti plasmid

Ti plasmid of *Agrobacterium* was isolated by using Alternative Boiling by Miniprep method. Then further, isolated Ti plasmid was separated and analyzed by 1% agarose gel electrophoresis and band was observed as shown in Figure 5. From the observation, it was confirmed that, this isolated *Agrobacterium* species harbored Ti plasmid. Molecular weight was evaluated by using DNA ladder. which was 500 bp molecular weight. The molecular weight of the *Agrobacterium* Ti plasmid below the 500 bp.

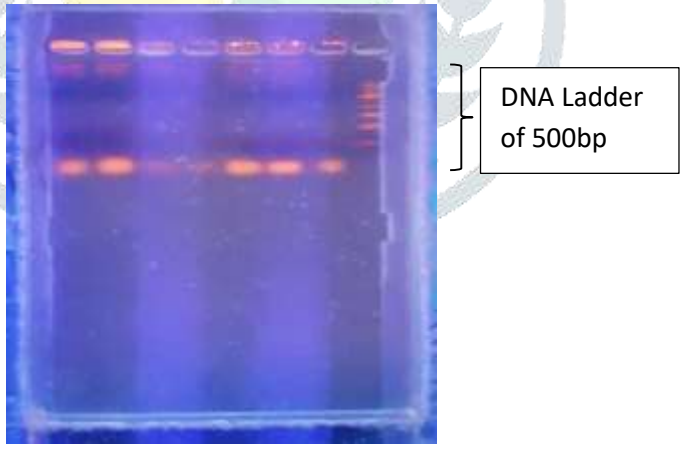

Figure 5: Plasmid isolation

Temperature Optimizations for growth of *Agrobacterium* isolate:

Agrobacterium isolate was grown on different temperatures as 25ºC, 37ºC, 40ºC. Isolate shown heavy growth at 25ºC and 37ºC as medium became more turbid as compared to control which placed at room temperature, whereas isolate showed very slight growth at 40ºC as shown in Figure 6.

Figure 6: Agrobacterial growth at various temperatures

Investigation of Ti plasmid stability at different temperatures

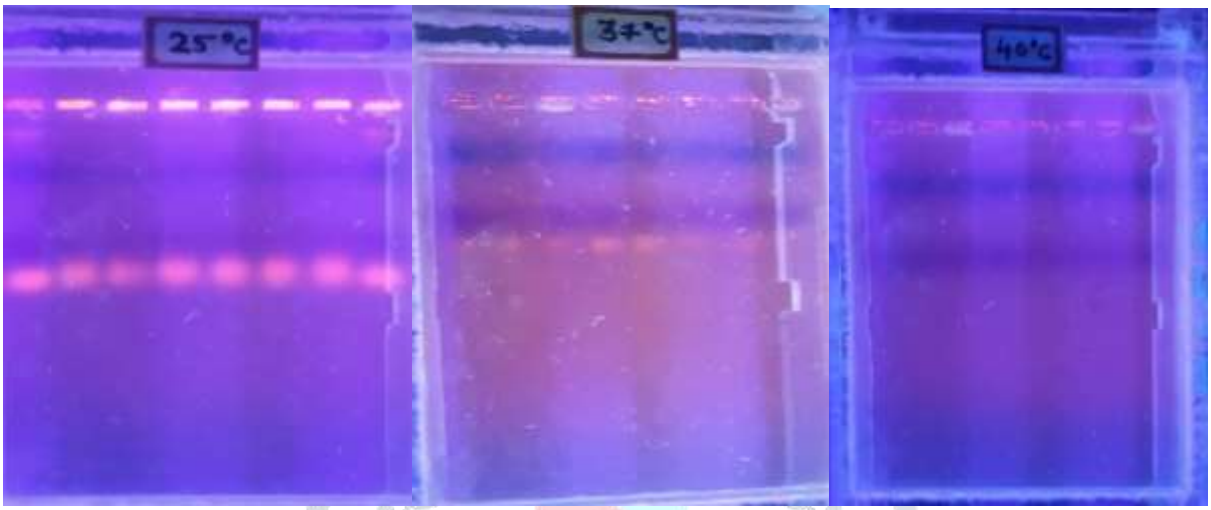

Figure 7: Isolation of Ti plasmid from *Agrobacterial* isolate grown at 25ºC

The Ti plasmids were isolated from isolate that grown on different temperatures as 25ºC, 37ºC, 40ºC. Effect of various temperatures (25ºC, 37ºC and 40ºC) on growth of *Agrobacterium* and its plasmid was checked and it was found that high temperature (40ºC) affected growth of *Agrobacterium* but had no effect on plasmid (Figure 7).

RFLP (Restriction Fragment Length Polymorphism) analysis

Ti plasmid also isolated from standard *Agrobacterium tumefaciens* strain 2146. Standard Ti plasmid and isolated *Agrobacterium's* Ti plasmid were digested by using restriction enzymes Hind III, EcoR1 (Figure 8). But fragments were not observed, these results showed that may be restriction site was absent in isolated Ti plasmid.

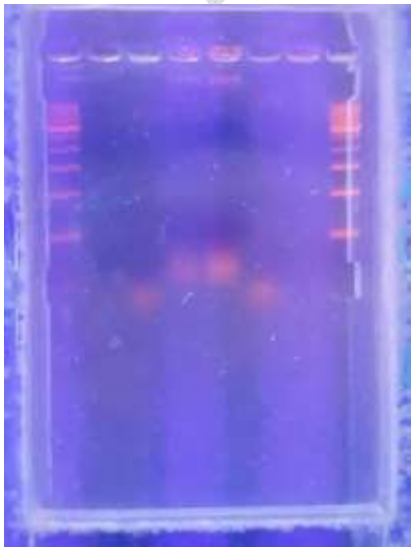

Fig 8: RFLP analysis

IV. Conclusion

The present study concluded that the existence of *Agrobacterium* is very predominant in the area of Rhizosphere soil. Some strains of *Agrobacterium* are pathogenic and some are nonpathogenic but strains may become virulent by transferring the Ti plasmid into nonpathogenic strain and that's very important nature of *Agrobacterium*. Our study showed that *Agrobacterium* is sensitive to some antibiotics such as Cefotaxime, Streptomycin so that antibiotics may be used for the prevention of Agrobacterial infection which leads to increase in economic value. Growth of *Agrobacterium* is quite excellent as they are capable to grow at temperature up to 37ºC. The present study also reveals that Ti plasmid was stable at temperature 25ºC and 30ºC but not stable at high temperature as 40 ºC. RFLP analysis was also done by using restriction enzymes Hind III and Eco R1 but no fragments were observed whereas intact band revealed that Ti plasmid may not contain restriction sites for these enzymes that presently analyzed.

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