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Isolation and identification of bacterial pathogens from cotton crop grown in Alwar district of Rajasthan, India

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ABSTRACT
Gossypium species on the economic point of view has significant values in agriculture field of all countries. Cotton is rich sources as foodstuff, fiber, feed, oil and biofuel worldwide. However, the economic values of cotton are continuously reduced due to pathogenic infection on cotton plant. Various symptomatic diseases on cotton plant have been reported throughout the world. Among them Verticillium wilt, Alternaria leaf spot, Fusarium wilt, bacterial blight, boll rot, leaf curl disease and seedling disorders are main responsible diseases to restraints the production level of cotton fiber. Therefore, in this research, isolation and identification of bacterial in cotton in Rajasthan were done. The cotton samples were collected from Mundawar, Behror and Thanagazi area of Alwar District. Overall, 7 bacterial isolates were detected from all areas in which 2 in Mundawar (yellow and white lint), 2 in Behror (yellow and white lint), and 3 other isolates of Thanagazi were green lint, white lint according to morphological identification. Furthermore, on the basis of biochemical analysis three Pseudomonas species: Pseudomonas aeruginosa, Pseudomonas syringae and Pseudomonas viridiflava were identified.
Keywords: *Cotton, Pseudomonas aeruginosa, Pseudomonas syringae, Pseudomonas viridiflava.*

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INTRODUCTION

Cotton is an essential natural source of human being from over the past decades to their live hood [13, 38]. It has sixth rank as vegetable oil production as well as broadly used as naturally production of fiber [12]. On the basis of cultivation it has globally seventh ranked and genetically modified cotton crop is the most planted GMO crop worldwide [23]. In Rajasthan cotton plant is cultivate approximately 5.03% area and total production occurs almost 2.89%. Northern and south-eastern area are highly cultivate region of Rajasthan state. For instance Alwar, Pali, Chittaurgarh, Ganganagar, Udaipur, Ajmer, Bhilwara and Jhalawar are main farm land of cotton. Desi cotton, Raj-Amer cotton, DCH cotton, BT-cotton, Malvi/Punj-Amer cotton, Shanker-4/6 cotton and Rajasthan American etc. are principal cotton varieties. In India, agriculture growth and instability has remained subject of intense debate in the agricultural economics literature [34]. Cotton belongs to Gossypium genus and Malvaceae family can be distributed into wild and cultivated cotton. A total of fifty cotton species are reported in which four are cultivated cotton and forty six are growing as wild cotton in the tropical and sub-tropical area [14, 7]. Cotton is perennial crop plant but now it is annually cultivated in several countries. Cotton bud is widely useful part that utilized in various products such as paper, textiles, drugs, edible oil and livestock feed [17, 31, 32, 10]. Cotton plant is rich sources of bio-active compounds including carbohydrates, proteins, fatty acids, terpenes, phenolics and lipids [3, 33, 21, 9]. Cotton stems, stalks, roots, bolls, leaves, seeds and calyx have these active compounds in higher concentration [21, 16, 22, 30].

Globally *Gossypium* species are used to production of oil, biofuel, fiber, feed and foodstuff. Approximately 95% cotton sources relates to *G. hirsutum* (upland cotton). Though, cotton's production is declined due to numerous biotic stresses. Pathogenic diseases in cotton plant causes reduction in crop yield by effecting seed germination and plant mortality, decreasing plant growth and quality of cotton lint. Cotton leaf curl, fungal wilt, bacterial rust, root-rot, anthracnose and bacterial wilt are significant names of cotton diseases in which bacterial blight is most occurring disease of cotton that causes by *Xanthomonas citri* pv. malvacearum. *Xanthomonas citri* pv. malvacearum affects each vegetative and reproductive stages of cotton plant. There are no actual chemical treatments present that can use in the cotton field to prevent bacterial blight (Cox *et al.,* 2019). The bacterial phytopathogen *P. syringea* belongs to the class of

Gammaproteo bacteria infect more than 80 plant species and this reduces production while also affecting fruit quality. Therefore, in the present study isolation and identification of pathogenic bacterial strains were carried out from infected cotton plant parts in Alwar district of Rajasthan. The bacterial isolates were identified by morphological and biochemical tests.

MATERIAL AND METHODS

A. COLLECTION OF EXPERIMENTAL SAMPLES

The infected plant materials (seed and fruit) were collected from the Thanagazi, Behror and Mundawar area of Alwar district of Rajasthan from the farmer's field.

B. ISOLATION OF BACTERIA

Nutrient agar (NA) media was prepared and small parts of all infected plant samples (seed and fruit) were put on NA plate individually in triplicate form and kept the plates for incubation at 37°C for 24h. After incubation, some different types of bacterial colonies were observed in all sample plates. These different bacterial colonies were pick out very carefully from each sample plate and sub-cultured on fresh NA plates. All sub-cultured plates were again incubated at 37°C for 24h to get every bacterial pathogen separately. These isolated bacterial pathogens were observed on the basis of morphological and biochemical characterizations.

C. MORPHOLOGICAL CHARACTERIZATION OF BACTERIA

The isolated bacterial pathogens were initially identified by morphological studies and Gram's staining method.

a. **Gram's staining**

Hildebrand and Schroth, 1972 method was used to Gram's staining method. The observation was done by using microscope. Red color (stained with safranine) denotes presence of gram negative bacteria and blue or purple color (stained with crystal violet followed by Iodine solution) for gram positive bacteria.

D. BIOCHEMICAL TEST FOR BACTERIAL ISOLATES

a) Catalase test: A drop of 3% hydrogen peroxide (H_2O_2) was added into each bacterial slant. Bubbling of oxygen gas indicates catalase positive and gram negative bacteria and no bubbling denotes catalase negative and gram positive bacteria [11].

b) KOH test: Jaya Chandra and Subha Mani, [25] method was used to differentiate gram-positive and gram-negative bacteria. Inoculum of bacterial isolates culture was added into a drop of 3% potassium hydroxide (KOH) solution on a clean glass slide. Gram-positive bacteria did not changed the viscosity of the KOH solution while Gram-negative bacteria caused the KOH solution to become stringy or mucoid in appearance and consistency.

c) Oxidase test: The modified Kovac's, [27] protocol was followed to identify oxidase activity of isolated bacteria. Took 3 pieces of filter paper for each pathogenic bacterium and properly labelled it. Rubbed an inoculating loop of each isolated culture on the Kovács oxidase reagent moistened filter paper. Within 5 to 10 sec. a deep purple-blue or blue color was visible that indicates positive result of oxidase production and no color change showed negative results?

d) Indole production test: Indole production test was performed by following modified MacFaddin, [28] method. 1% tryptone broth was prepared and inoculated with isolated bacterial culture. All inoculated the tubes were incubated tentatively for 48 hrs at 37°C. After incubation 5 drops of Kovac's Reagent was added into each tube and shake gently after 10 minutes of interval. A ring of cherry red color was appeared on the surface indicates a positive result. Appearance of yellow ring indicated negative result.

e) Citrate utilization test: Modified Jawetz *et al.,* [24] 9method was used to identify citrate utilization potential of bacterial isolates**.** The slant of Simon's citrate agar media was prepared by pouring 5ml of media in each tube. After 48 hrs of incubation at 37°C, green color of media was changed into blue color indicates positive result. No change in indicated negative results.

f) Cetramide test: A modified method according to Brown and Lowbury [5] cetrimide agar media was prepared and then add 5 ml media was poured into each tubes followed by prepared slants. Thereafter each tube was inoculated with isolated bacterial culture. In tubes dark blue-green color of bacterial growth will observe show positive result and no growth will observe means negative result.

Confirmatory test

g) Gelatin hydrolysis: A method of gelatin hydrolysis was performed by using Elliott and Stead, 1987 method with some modifications. Gelatin Media was prepared and stab inoculation was done using inoculating loop of each bacterial isolates. The inoculated tubes were observed for liquefaction after incubation for 4-7 days at 37°C. All tubes were allowed to stand for 30 minutes in refrigerator at 4°C. The liquefied media indicated positive result and solidified media indicated negative result.

h) Arginine Dihydrolase Broth test: Elliott and Stead, [8] method with some modifications was applied to test arginine dihydrolase potential of bacterial isolates**.** Inoculated arginine dihydrolase broth was incubated at 37°C for 48 hrs. Tentatively. Positive results of arginine dihydrolase test were observed on the basis of color change from purple color to light yellow color**.** Appearance of purple color indicated as negative results.

i) Carbohydrate fermentation test: Hemraj *et al.,* [18] method with some modifications was used to categorize carbohydrate fermentation potential of bacterial culture. 0.5% glucose was utilized in preparation of carbohydrate consumption broth. All inoculated broth was kept into incubator for 48 hrs. at 37°C. Purple color of media changed into yellow color gave positive results and denoted acid production during carbohydrate fermentation. No change in color indicated as negative results.

RESULTS AND DISCUSSION

The bacterial blight causes by *Xanthomonas* species is the most common disease of cotton crop . In bacterial isolation total 7 bacterial isolates were obtained in which two in Mundawar (yellow lint and white lint), two in Behror (yellow lint and white lint), and three other isolates of Thanagazi are green lint, white lint and Thanagazi seed from infected cotton parts. Bacterial isolates can be gram-positive and negative cells, however the cells of isolates were gram-negative and rod shaped in this study. To determine the genera of bacterial isolates, some of biochemical tests were performed. There are results of morphology test and some of biochemical tests were shown in table 1 and 2. In 1946, Smith recognized gram-negative bacteria with rod shaped, nonacid fast bacteria and motile bacteria due to presence of 1 or 3 polar flagella of *P. lachrymans* pathogen in morphological studies. A study conducted by Kagiwata, [26] observed gram-negative with straight rod shaped, non-sporing, aerobic and motile with 1-5 polar flagella in morphological studies of various isolates, which revealed as *P. syringae* pv. Lachrymans. Bradbury, [4] described morphological characterization of Pseudomonads in which it established as gram negative, curved/straight, 0.5-1.0 × 1.5-4.0 μm bacterial size and aerobic with rods shaped. Shila *et al.,* [35] reported creamy white color of bacterial pathogen isolated from various infected cucurbit seeds and this isolate was recognized as *Pseudomonas* species by using both solid and liquid media.

Biochemical tests were performed on the 7 bacterial isolates identified as Gram-negative, rod-shaped. Behror white lint, Thanagazi green lint, and Thanagazi seeds were showed citrate utilization test and citramide positive it means *Pseudomonas aeurginosa* was present. Further the confirmatory of *Pseudomonas aeurginosa* those were citramide positive they again tested with Gelatin hydrolysis and Arginine hydrolysis and found that *Pseudomonas aeurginosa* was confirmedly present in three bacterial isolates of Behror white lint, Thanagazi green lint, and Thanagazi seeds. Those were citramide negative such as Mundawar (Yellow and White lint), Behror yellow and Thanagazi white lint further test with carbohydrate fermentation to check the presence of another *Pseudomonas* species and results indicated that Mundawar yellow lint isolate was carbohydrate fermentation positive, found that *Pseudomonas syringae* and negative results showed presence of *P. viridiflava* pathogen that recognized in Mundawar white lint, Behror yellow lint and Thanagazi white lint isolates. KOH test is easiest and quick method to differentiate gram-positive and gram-negative bacteria in comparison to gram's staining method according to the Suslow *et al.,* [37]. As similar to bio-chemical results of *P. syringae*, Aksoy, [2] revealed catalase positive and negative result of arginine dihydrolase, which recognized as *P. syringae* gram-negative bacteria. Similarly in biochemical assay of Shila *et al.,* 2013 study, gram negative results of Gram's staining, KOH test and Kovac's oxidase test, and positive of carbohydrate utilization and catalase test etc. were observed according their results *P. syringae* were detected. Habiba *et al.*, [15]; Msogoya *et al.*, [29] characterized *Cellulomonas* species, *Klebsiella* species, *Corynebacterium* species, *Proteus* species, *Bacillus* species, *Erwinia* species and *Staphylococcus* species. Smith, [36] revealed positive results of ammonia production and negative results of H_2S and indole production, starch hydrolysis and nitrate reduction test. Furthermore, utilization of laevulose, sorbitol, sucrose, arabinose, mannose, xylose, galactose and dextrose were also observed. According to these bio-chemical results *P. lachrymans* was recognized. Pseudomonas is circular in shape and has cream-yellowish color. Winarni, [39] also characterized *Pseudomonas* species from infected seeds of soybean and rice by successfully observation of morphological and bio-chemical results. Consequently, the morphological and biochemical results of this study confirmed the infection of *Pseudomonas* species in infected cotton plant.

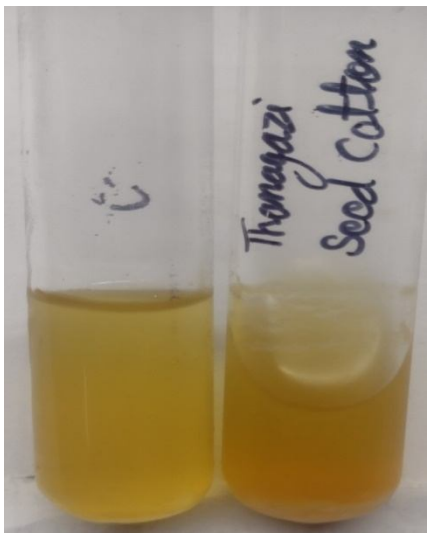

Figure 1: Gelatin hydrolysis test for bacterial isolate

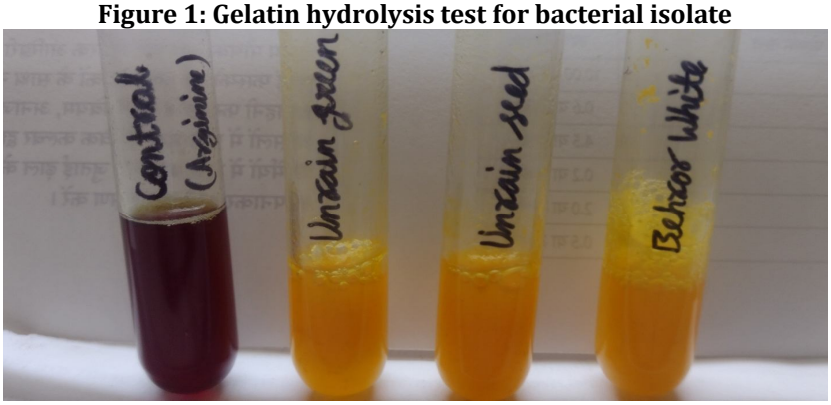

Figure 2: Arginine dihydrolase test for bacterial isolate of Umrain (Thanagazi) green lint, Umrain (Thanagazi) seed, Behror white lint

Figure 3: Carbohydrate fermentation in glucose test for bacterial isolates Mundawar yellow lint [Positive results]

Table 1: Morphological Characterization of Bacteria

Bacterial isolates	Color	Shape	Opacity	Gram-staining	Cell form
Mundawar yellow lint	Light-yellow	Irregular	Undulate	negative	rod shaped
Mundawar white lint	White	Irregular	Lobate	negative	rod shaped
Behror white lint	White	Irregular	Lobate	negative	rod shaped
Behror yellow lint	Yellow	Raised irregular	Undulate	negative	rod shaped
Thanagazi green lint	Green	Raised irregular	Curled	negative	rod shaped
Thanagazi white lint	White	Raised irregular	Entire	negative	rod shaped
Thanagazi seeds	White	Raised irregular	Lobate	negative	rod shaped

Table 2: Biochemical Test for Bacterial Isolates

Infected Sample	Catalase test	KOH test	Oxidase test	Indole production	Citrate utilization	Citramide test	Gelatin hydrolysis	Arginine dihydrolase	Carbohydrate fermentation	Bacterial isolates
Mundawer yellow lint	+	+	-	-	-	-			+	*Pseudomonas syringae*
Mundawer white lint	+	+	-	-	-	-			-	*Pseudomonas viridiflava*
Behror white lint	+	+	+	-	+	+	+	+		*Pseudomonas aeruginosa*
Behror yellow lint	+	+	-	-	-	-			-	*Pseudomonas viridiflava*
Thanagazi green lint	+	+	+	-	+	+	+	+		*Pseudomonas aeruginosa*
Thanagazi white lint	+	+	-	-	-	-			-	*Pseudomonas viridiflava*
Thanagazi seeds	+	+	+	-	+	+	+	+		*Pseudomonas aeruginosa*

"+" = Positive Reaction, "-" = Negative Reaction

CONCLUSIONS

Cotton is an economically important crop in the world they need to be protected from harmful diseases. The morphological and biochemical results of the present study concluded the infection of *Pseudomonas* species: *P*seudomonas *aeruginosa, Pseudomonas syringae* and *Pseudomonas viridiflava* in infected cotton plant. Therefore, further investigations are essential to identify the detailed information related to the identity and mode of mechanisms of pathogens to infect the healthy plants. The sustainable management by bio-fertilizers is also necessary to inhibit bacterial infection on plants.

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