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Research Article

IDENTIFICATION, DISEASE TRANSMISSION AND PHYTOPATHOLOGICAL EFFECTS OF BACTERIAL PATHOGENS ASSOCIATED WITH *VICIA FABA*

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ABSTRACT

Symptomatic infected *Vicia faba* L. (Fava bean) plant parts were collected from three districts (Alwar, Bundi, and Kota) of Rajasthan to study the presence of bacterial pathogens. A total of 11 pathogenic bacteria isolated were on the basis of morphological studies such as color, shape and opacity. In biochemical analysis, out of 11 isolates, 2 bacterial isolates showed gram negative, rod shapes, catalase positive, KOH positive, oxidase positive, indole production negative, citrate utilization positive, cetrimide positive, gelatin hydrolysis positive and arginine dihydrolase positive results of *Pseudomonas aeruginosa*, 8 isolates showed gram negative, rod shapes, catalase positive, KOH positive, oxidase negative, indole production negative, citrate utilization negative, cetrimide negative, carbohydrate fermentation positive results of *Pseudomonas syringae* and 1 isolates gave gram negative, rod shapes, catalase positive, KOH positive, oxidase negative, indole production negative, citrate utilization negative, cetrimide negative, carbohydrate fermentation negative results of *Pseudomonas viridiflava*.The shriveling, rotting, white bacterial growth on and around seed-borne inoculums and less germination rate was observed in disease transmission experiment. Additionally same pathogens were detected in transmission methods that revealed the transmission of bacteria from one generation to other by vertical transmission. Artificially infected seeds in phytopathogenecity test also caused the reduction in germination, reduced shoot-root lengths and blacking of radicle.

INTRODUCTION

Legumes are important food because of their high nutritional value, ease of preservation and their low cost of production (Rahate *et al.,* 2021).Legumes are a good source of protein, dietary fiber, low glycemic indexes, lower levels of fat (2–5%) and high amount of carbohydrate (Xu *et al.,*2007). Among them, *Vicia faba* L. (Fava bean) is one of the cheapest protein sources in most of the developing countries like Africa, Latin America and Asia (Ali *et al.,* 2014a; 2014b; Duc, 1997). Fava bean is a winter grown legume which belongs to the family Leguminosae. Mediterranean countries, Egypt, Ethiopia, China, India, Afghanistan, Northern Africa and Northern Europe are the major fava bean producing countries (Rahate *et al.,* 2021).Fava beans have proteins (36–39%), starch (42–47%) (Alghamdi, 2009), minerals, vitamins (Jezierny *et al.,* 2010), dietary fiber (8%) and ash level (3.5-4%). It is rich sources of folate, choline, lecithin (Coda *et al.,* 2015), secondary metabolites and bioactive compounds, such as antioxidants, phenols and γ-aminobutyric acid (GABA) (Khazaei *et al.,* 2019).The role of fava bean contributes in food and feed industry to increase its nutritional quality and benefit to human health. In addition, fava beans are also a good source of minerals like iron and zinc. In China, plant foods provide at least 50% of the dietary energy and nutrients, and fava bean is one of them. Fava beans are a good source of L-dopa, a precursor of dopamine, is used as a drug for the medication of Parkinson's disease (Rabey *et al.,* 1992).Besides, positive effects of fava beans are reduction in plasma LDL-cholesterol levels (Ma *et al.,* 2005).

Pathogenic infections such as molds, viruses, fungi, nematodes, and bacteria produce adverse effects on plants. Bacteria, single-cell microscopic pathogens, cause detrimental effects on plants. Approximately 200 bacteria are identified that reduce the plant's productivity for several years (Leonberger *et al.,* 2016). Commonly pathogenic bacteria belonging to *Enterobacteriaceae*, *Xanthomonadaceae*, and *Pseudomonadaceae* families infect all plant species to nutrition and habitat. The main harmful genera of plant-bacteria are *Burkholderia, Pantoea, Spiroplasma, Ralstonia, Clavibacter, Phytoplasma, Erwinia, Acidovorax, Pectobacterium, Xylella, Streptomyces, Agrobacterium, Pseudomonas,* and *Xanthomonas* (Kannan *et al.,* 2015).Plant bacterial diseases are

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generally characterized by plant morphological symptoms such as leaf and fruit spots, cankers, blights, vascular wilts, rots, and tumors (Buonaurio, 2008).Microbial pathogenicity has often been defined as the biochemical mechanisms whereby pathogenic microorganisms cause disease in a host organism (Fuchs, 1998). Pathogenicity of Gram-negative plant pathogenic bacteria are strictly dependent on the presence of secretion apparatuses in host cells, through which they secrete proteins or nucleoproteins involved in their virulence within the apoplast or inject these substances into host cells (Buonaurio, 2008). Bacterial pathogenicity depends upon bacterial secretion systems, quorum sensing (QS), plant cell-wall-degrading enzymes, toxins, hormones, polysaccharides, proteinases, siderophores, and melanin.The goal of the present study was to identify pathogenic bacteria responsible to cause and yield losses in *Vicia faba*. The study aso aimed to observe seed to seedling transmission and phythopathological effects of isolated and identified bacterial pathogens.

MATERIAL AND METHODS

Plant material

The infected *Vicia faba*plant parts (seeds, leaves, and fruits) were collectedfrom the farmer's field of three districts of Rajasthan (Alwar, Bundi, and Kota). The plant materials were taxonomically identified and authenticated by Department of Botany, University of Rajasthan, Jaipur.

Isolation of Bacteria from Samples

Nutrient agar (NA) media was prepared and small parts of all infected plant samples (seeds, leaves, and fruits) were put on NA plate individually in triplicate formand kept the plates for incubation at 37°C for 24hrs. After incubation, some different types of bacterial colonies were observed. These various 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 24hrs to get individual bacterial colonies. These isolated bacterial pathogens were observed on the basis of morphological and biochemical characterizations.

MORPHOLOGICAL CHARACTERIZATION

The isolated bacterial pathogens were initially identified with morphological characters (Gram's staining methodand KOH test). Morphological studies were done based on color, shape, and opacity.

Gram's staining

A thin smear of isolated bacterial culture was prepared on a slide and then gram staining was performed by following Hildebrand and Scroth, 1972 method. The slide was air-dried and fixed it using heat. The bacterial smear was flooded with crystal violet and left for 1minute. The slide was washed properly with deionized water. The slide was flooded with gram's iodine solution and allowed to stand for 30 s, thereafter the slide was washed completely with deionized water. For decolorization, the slide was flooded with 95% ethyl alcohol and washed with distilled water. Further, the smear was covered with safranin for 20 seconds and washed with distilled water. In the end, air-dried the slide was, and then observation was done under a compound microscope (10-40X).

KOH test

Gram-positive and negative bacteria can also differentiate by the KOH procedure, as described by Jaya Chandra and Subha Mani, 2011.More inoculum of isolated bacterial culture was added into a drop of 3% aqueous solution of KOH on a cleaned glass slide and mixed properly by inoculation loop. The glass slide was cleaned with 70% ethanol. It was stirred completely up to approximately 1 minute and then the loop was lifted slowly to observe the string. The string was seen within the first 30 seconds after mixing in the KOH solution.

BIOCHEMICAL CHARACTERIZATION

Catalase test

The catalase activity in isolated bacteria was identified by using Facklam and Elliott, 1995. NA slant was inoculated with isolated bacterial pathogens. Thereafter the tubes were incubated at 37°C for 24 h. After incubation 3-4 drops of 3% hydrogen peroxide were added to the growth of each slant. The culture was observed for the presence or absence of gas bubbles.

Oxidase test

Modified Kovac's, 1956 procedure was used to detect cytochrome c oxidase potential in bacterial isolates. Took 3 pieces of filter paper for each pathogenic bacterium and labeled them properly. Each isolated culture was rubbed by a sterile inoculation loop on the filter paper moist with Kovács oxidase reagent. Thereafter filter paper was observed to check the color of the smear after 15 to 30 seconds of rubbing. Oxidase-positive bacteria show dark blue color within 5 to 10 seconds.

Indole production test

Indole test was done by using a modified MacFaddin, 2000 method. 1% tryptone broth was prepared and sterilized by using an autoclave. The tryptone broth tubes were inoculated individually with each isolated bacteria and incubated at 37°C for 48h. 5 drops of Kovac's reagent were added into each tube after 48h of incubation. The tubes were shaken gently after 10-minute intervals. Thereafter the tubes were left for a few minutes to prepare a layer of reagent on the top. The observation was done based on color. Indole positive bacteria form a cherry red-colored ring on the surface of the media and indole negative bacteria forms a yellow ring.

Citrate utilization test

Jawetz *et al.,* 1989 method with some modification was used to citrate utilization test. Simon's citrate agar media was prepared and sterilized by using an autoclave machine. Slants were prepared by pouring 5ml of media into each tube. After slants preparation, each tube was inoculated with isolated bacterial culture and then incubated for 48hrs. at 37°C. At the end of the incubation period based on growth and color, the tubes were observed. In citrate positive result, the color of media changes from green to bluish, and in negative result color remainsthe same as the control.

Cetrimide test

A modified method according to Brown and Lowbury (1965) was used to observe cetrimide positive and negative results. Cetrimide Agar Media was prepared and heated to mix the medium properly. After proper dissolving, media was autoclaved for sterilization. 5 ml sterilized media was carefully

poured into each tube followed by prepared slants. Thereafter each tube was inoculated with isolated bacterial culture, followed by incubation for 48h at 37°C. After 48 h of incubation, all tubes were properly observed based on color and growth. The dark blue-green color of bacterial growth was observed as cetrimide positive bacteria and no growth was denoted as cetrimide negative bacteria.

Gelatin hydrolysis

Gelatin hydrolysis tests done by using the modified Lelliott and Stead, 1987 method. Gelatin media was prepared and autoclaved at 15 lbs pressure (121°C) for 15mins. Performed stab inoculation using an inoculating loop, from each isolated bacterial culture into labeled test tubes. All inoculated and un-inoculated (control) tubes were incubated at 37°C for 4 to 7 days. After incubation, place the tubes in the refrigerator at 4°C at least for 30 min. or until the control tube solidifies. The inoculated tubes were observed for liquefaction. The liquefied media showed positive results and solidified media showed negative results.

Arginine Dihydrolase test

Modified Lelliott and Stead, 1987 was used for the arginine dihydrolase broth test**.**Arginine Dihydrolase Broth medium was prepared and heated if necessary to dissolve the medium completely. 5ml of broth was distributed into each tube. All tubes were allowed to cool in an upright position. Then each tube was inoculated with isolated bacterial culture and incubated for 48hat 37°C. Purple-colored converted into light yellow color referred to as positive result and no change in color noted as negative results.

Carbohydrate fermentation test

Modify Hemraj *et al.,* 2013method was performed to identify carbohydrate fermentation activity in isolated bacterial culture.16.1 grams above reagents dissolved in 990 ml distilled water and heated for properly dissolving all reagents in distilled water. This prepared broth was autoclaved (121°C, 15 lbs pressurefor 15 minutes) for sterilization. Thereafter aseptically added 10 ml separately sterilized carbohydrate (glucose) solution to give a final concentration of 0.5% and mixed completely. Each tube containing a specific carbohydrate broth medium was labeled and inoculated with isolated bacterial culture and incubated at 37°C for 48h. All sample tubes then were compared with control based on color change from purple color (transparent) to yellow color (translucent) [due to production of acid]. The purple color of the broth medium changed into a yellow color that indicated acid production by isolates due to fermentation of glucose referred to as positive bacteria.

Disease Transmission and Phyto-pathological Effects

Two types of naturally infected *Vicia faba* L.seeds carrying 80% and 89% infection were selected to disease transmission studies. The experiment was done in triplicate forms. 30 seeds per infected sample were placed at equal distances on a moist cotton bed according to 10 seeds per plate. In the pot experiment, 30 seeds per infected sample were placed in plastic pots according to the 5seeds per pot with maintaining a specific distance. All plates and pots were incubated at 25±2°C under 12h of alternating cycles of artificial light and darkness for 7 days. At the end of incubation, germination of seeds in percentage was recorded. Thereafter infected parts of seedlings were used to isolate pathogenic bacteria and the identification of bacterial pathogen was done by using above mentioned biochemical methods (Jain and Agrawal, 2011).

To studyphyto-pathological effects, the healthy fresh fava bean seeds were artificially treated with isolated bacterial broth culture and were sown on moist cotton beds and incubated for 7 days under the above described optimum conditions. Thereafter seedlings were again artificially treated with isolated bacterial broth culture and performed blotter method and incubated for up to 7 days. The effects of pathogenicity on seeds and seedlings were identified based on percent seed germination rates, and length of shoot and root by comparing with un-treated seeds (Jain and Agrawal, 2011).

RESULT AND DISCUSSION

Morphological Characterization

A total of 11 bacterial colonies were isolated on the basis of color, shape, and opacity as specified in Table 1. The isolates showed white, off-white and pale-white in color, regular, raised irregular and flat irregular in shape and filiform, undulate filiform, lobate, and filamentous scalloped types opacity. In order to characterization, gram staining and KOH test were performed in which gram negative bacteria were observed. Under microscopic studies, all isolates were rod shaped. In morphological analysis of Sharma, 2017, circular, convex to domed, mucoid, entire, shiny and raised bacterial colonies of *Xanthomonas axonopodis* pv. vesicatoria with yellowish color was recognized on yeast dextrose carbonate agar medium. Yousif *et al.,* 2018 isolated bacterial colonies from devastating wilt disease of guar that produced mucoid yellow colonies on nutrient agar and yeast dextrose carbonate media.The bacteria isolated from soybean leaves in the Sain and Gour, 2013 studies showed smooth, yellowish, shining colonies with convex elevation and entire margin on nutrient agar media.The isolates were gram-negative with rods, one polar flagellate, non-sporing, negative acid-fast test, 0.9 x 1.6 µm size that was similar morphology of *Xanthomonas* genus. *Xanthomonas*genus gave yellow color due to production of xanthin and rod shape, medium size, convex elevation, even margin and mucoid surface in the Haider *et al.,* 2020 morphological studies.

Biochemical Characterization

The identification of bacterial isolates in this study was carried out on the basis of bio-chemical analysis. All isolates gave catalase positive results with bubbling of free oxygen gas. Among the 11 isolates, 2 isolates (seed 1 and leaf dry 2) showed blue color of oxidase positive. Indole test was negative of all bacterial isolates. Seed 1 and leaf dry 2 bacterial isolates gave positive results of citrate utilization and cetrimide method. These isolates also exhibited gelatin hydrolysis and arginine dihydrolase activity. Other 9 isolates showed oxidase negative, citrate negative and cetrimide negative results. Furthermore, the positive results of carbohydrate fermentation test was observed with seed 2, leaf dry 1, fruit dry 1, fresh fruit 1(1), fresh fruit 1(2), fresh fruit 1(3), fresh leaf 2, and fresh leaf 3 and negative with fruit dry 2isolates.According to these biochemical results, seed 1 and leaf dry 2 can be referred as *Pseudomonas aeruginosa*, isolates of seed 2, leaf dry 1, fruit dry 1, fresh fruit 1(1), fresh fruit 1(2), fresh fruit 1(3), fresh leaf 2, and fresh leaf 3 canbe recognized as a *Pseudomonas syringae* and isolates of fruit dry 2 canbe identified as *Pseudomonas viridiflava*.

Isolates of Sharma, 2017 studies gave gram's negative, catalase positive, KOH positive, Kovac's oxidase negative, levan negative,starch hydrolyzing, lipase activity positive, arginine variable, gelatin hydrolyzing and no rotting of potato tissue. According to their bio-chemical results, bacterial colonies were recognized as*Xanthomonas axonopodis.* Yousif *et al.,* 2018 isolated three bacterial strains from wilt disease of guar that were gram negative, catalase positive, oxidase positive, motile, starch hydrolysis, gelatin hydrolysis, glucose and manose utilization positive bacteria and recognized as *X. axonopodis*. Haider *et al.,* 2020 characterized *Xanthomonas campestris* by biochemical analysis on the basis of positive results of starch hydrolysis test, H_2O_2 test, Simmons citrate test, KOH test, methyl red/ voges-proskauer, gelatin hydrolysis test, casein test and negative results of oxidase and nitrate reduction test. By bio-chemical characterization Sain and Gour, 2013 recognized *Xanthomonas*genus in which bacterial isolates gavenegative results of KOH, indole production,bile tolerance, oxidase, methyl red/ voges-proskauer, arginine dihydrolase and nitrate reduction test. Positive results of starch, casein and gelatin hydrolysis, H_2S production was observed.

Disease Transmission and Phyto-pathological Effects

Pathogenic bacterial strains are heterotrophic organisms that resist in plants in the form of parasites. Pathogens are unable to enter directly into plant cells by penetration. Natural surface openings such as nectaroides, hydatodes, stomata and wound region are main plant infection sites of pathogens. Among them leaf scars are significant region of infection. The symptomatic bacterial infections on plants are cankers, tumours, necroses, soft rots, blights and wilting (Sobiczewski, 2008). In petri-plate method, 50% germination rate was observed with 89% infected fava bean seeds and 80% seed germination with 80% infected seeds in the present study. The non-germinated seeds showed shriveling, rotting, and one seed of 89% infected fava-bean seeds completely covered with white bacterial growth with rotting. However, the radicles of germinated seeds were also dried and became blackish within 7 days. In pot experiment, 30% germination was recorded with 80% infected seeds and 10% germination with 89% infected seeds. Accordingly 70% and 90% mortality was observed with 80% and 89% infected seeds, respectively. Some non-germinated seeds were shriveled and rotted. Green and white bacterial growth also observed on and around the other non-germinated seeds. The infected radicle and plumule was used to identify bacterial strains by using bio-chemical analysis. Bio-chemical characterization again *P. aeruginosa*, *P. syringae*and *P. viridiflava* isolates were characterized on the basis of bio-chemical results. It was observed that disease is transmitted in next plant generation by seed borne inoculum. A study conducted by Sharma, 2017 showed 14.39% and 27.01% mortality of highly infected chilli seeds of ac. nos. Ca-1227 and Ca-1234. Non-germinated chilli seeds exhibited rotting with heavy pale-cream to yellow oozing of the bacterium on and around the seeds and browning and rotting of seedlings. The symptomatic seedlings showed browning of radicle and plumule and blighted lesions on cotyledonary leaves which later on showed rotting and bacterial oozing in disease transmission method of Jain and Agrawal, 2011.

Table 1 Morphological and Bio-chemical Characterization of isolated pathogenic bacteria

Infected Sample	Color	Shape	Opacity	Gram staining	Catalase test	KOH test	Oxidase test	Indole production	Citrate utilization	Cetramide test	Gelatin hydrolysis	Arginine dihydrolase	Carbohydrate fermentation	Bacterial isolates
Seed 1	White	Raised irregular	Filiform	Gram negative, rods	+	+	+	-	+	+	+	+		*Pseudomonas aeruginosa*
Seed 2	White	Raised irregular	Filiform	Gram negative, rods	+	+	-	-	-	-			+	*Pseudomonas syringae*
Leaf Dry 1	Off-white	Flat irregular (non-irregular)	Lobate	Gram negative, rods	+	+	-	-	-	-			+	*Pseudomonas syringae*
Leaf Dry 2	White	Flat irregular	Lobate	Gram negative, rods	+	+	+	-	+	+	+	+		*Pseudomonas aeruginosa*
Fruit Dry 1	Off-white	Raised irregular	Undulate filiform	Gram negative, rods	+	+	-	-	-	-			+	*Pseudomonas syringae*
Fruit Dry 2	White	Raised irregular	Lobate	Gram negative, rods	+	+	-	-	-	-			-	*Pseudomonas viridiflava*
Fresh Fruit 1(1)	Off-white	Flat irregular	Lobate	Gram negative, rods	+	+	-	-	-	-			+	*Pseudomonas syringae*
Fresh Fruit 1(2)	Off-white	flat irregular	Lobate	Gram negative, rods	+	+	-	-	-	-			+	*Pseudomonas syringae*
Fresh Fruit 1(3)	White	flat irregular	Lobate	Gram negative, rods	+	+	-	-	-	-			+	*Pseudomonas syringae*
Fresh leaf 2	Pale white	Regular	Lobate	Gram negative, rods	+	+	-	-	-	-			+	*Pseudomonas syringae*
Fresh leaf 3	Off-white	Flat irregular	Filament-ous scalloped	Gram negative, rods	+	+	-	-	-	-			+	*Pseudomonas syringae*

In phyto-pathological analysis, seed artificially treated with *P. aeruginosa*and *P. viridiflava*, individually showed 50% and 60% germination, respectively that were higher germination rates in comparison to the control (40%) but shoot-root length was reduced drastically. *P. syringae* treated seeds exhibited 40% germination with reduction of both shoot and root length.

Table 1 Morphological and Bio-chemical Characterization of isolated pathogenic bacteria

Sample	Control			*P. aeruginosa*			*P. syringae*			*P. viridiflava*		
	Seed germination (%)	Shoot length (cm)	Root length (cm)	Seed germination (%)	Shoot length (cm)	Root length (cm)	Seed germination (%)	Shoot length (cm)	Root length (cm)	Seed germination (%)	Shoot length (cm)	Root length (cm)
Seed treated	40	18.42	3.75	50	0.7	0.88	40	0.175	1	60	0.85	0
Seedling treated	40	18.9	3.88	50	1.04	1	40	0.425	1.02	60	1.10	0.52

The shoot and root length of control was 18.42cm and 4.75cm, respectively, while the range of shoot length of artificially infected fava bean seeds was 0.175-0.85, and 0.88-1cm range was observed with root length of treated seeds. No radicle was recorded with *P. viridiflava* infected seeds. After 7 days, seedling treated individually with *P. aeruginosa, P. syringae* and *P. viridiflava*and control showed slightly increment in shoot-root length but with pale greenish and white puffing growth around radicles when compared with control. The results suggested that all isolated bacteria are responsible to the seed viability and seedling growth. Jain and Agrawal, 2011 recorded 65.57% mortality in cluster bean seeds infected with *Xanthomonas axonopodis*. Artificially treated cluster bean leaves with *Xanthomonas axonopodis* exhibited yellowing and necrotic lesions starting from leaves tips. On inoculated fruits necrotic brown-sunken lesions with bacterial growth was observed. In 1991, Soni and Thind recognized occurrence of *X. campestris* on cowpea pods artificially infected. Pathogenicity test of Sharma, 2017 revealed 62.57% and 49.85% mortality in*X. axonopodis* infected chilli seeds of ac. nos. Ca-1227 and Ca-1234. Blight symptoms on seedling was observed with browning, rotting of radicle and hypocotyle. Artificially infection of *X. campestris* on seeds of cotton and *P. vulgaris*reported reduction of germination (Zochowski and Rudolph, 1991; Valarini and Menten, 1991). In the field experiment of Hulin *et al.,* 2018, *Pseudomonas* species showed pathogenicity by causing black/brown disease on plum.Emami*et al.,* 2020 firstly reported pathogenicity of *Pseudomonas putida* on eggplant, *Pseudomonas aeruginosa* on tomato and *Pseudomonas entomophila* on sweet pepper.

CONCLUSION

In the present study, infected *Vicia faba* plant parts such as seeds, leaves and fruits were categorized on the basis of shriveling, blackish color and black spots. Symptomatic plant parts yieldedbacterial colonieson nutrient agar medium that were characterized as *Pseudomonas aeruginosa*, *Pseudomonas syringae* and *Pseudomonas viridiflava*on the basis of morphological, biochemical analysis. Disease transmission studiesrevealed the transmission of bacterial pathogensin next generation of plant by seed borne inoculum with alleviate the germination and seedling growth. Phytopathological test was also described the reduction in seed viability. Consequently, the results concluded that bacterial infection on plant or plant parts are responsible to reduce the seed viability and plant growth.

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