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OUTBREAK OF BACTERIAL APICAL NECROSIS OF MANGO IN MULTAN, PUNJAB, PAKISTAN

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A B S T R A C T

Bacterial apical necrosis disease is the serious emerging problems in mango orchards of Punjab, Pakistan. The newly emerged twigs, inflorescence and leaves of the mango trees showed typical symptoms of the bacterial apical necrosis. The bacterium (*Pseudomonas syringae*pv. s*yringae*) was isolated on nutrient agar and purified on King's B medium from the symptomatic tissues. Biochemical characterization of the bacterium proved as gram negative having the ability to cause disease in plants. KOH test also countersigned the gram staining observations. Hypersensitivity reaction also showed the ability of pathogen to cause rapid necrosis of tissues within 24 hours of its inoculation. Survey showed the consistent occurrence of the disease in all the selected areas among and within the blocks irrespective of the mango cultivar. Maximum disease incidence was recorded on location-2 with 46.66% while minimum disease incidence was recorded on location-3 with 13.33%. Location-1 ranges from 25 to 41.66% disease incidence while location-2 ranges from 31.33 to 46.66% whereas location-3 ranges from 13.33 to 43.33% disease incidence. This work provides a description on new disease of mango tree in Pakistan which is found almost in every orchard of the mango belt of Punjab. This information will be the valuable tool to study the epidemiology and population genetic diversity of the bacterium in future.

Keywords: Necrosis, Buds, Bacterium, Black spot, Gram negative.

INTRODUCTION

Mango belongs to the family anacardiaceae and genus *Mangifera*, known as the king of all fruits due to its shape, color, taste and flavor. Mango is resident of South Asia where from it reached to the rest of world and hence, widely grown fruit in tropical and sub-tropical areas. Mango is the national tree of Bangladesh while it is national fruit of Pakistan. In Pakistan, Mango is specifically grown in the mango belt of Pakistan consisting of some Districts of Punjab and Sindh. It produces average yield of 11.20 tones/ hectare in Pakistan (Anonymous, 2015). Pakistan produces 1732 thousands tones of mango annually being the second major fruit crop of country to earn foreign exchange (Anonymous, 2014). For Punjab, Pakistan, Mango is considered its recognition for the whole world.

Mango trees are vulnerable to many diseases which are the main reason of its low production in Pakistan. Besides the many devastating diseases of mango, the bacterial apical necrosis disease is believed a dangerous pathosystem in core areas of mango production in world (Cazorla *et al*., 2006). Mango bacterial apical necrosis was discovered by Dr. Steve Akiew in Bundaberg; Queensland (District) in September 1999, after that in New South Wales and Byron Bay in December 1999 (Cazorla *et al*., 2006). The disease has also been identified in Israel, where it was known as 'bacterial black blight' (Pinkas *et al*., 1996), and then identified in Spain and Portugal, wherein it was termed as 'bacterial apical necrosis' (Cazorla *et al*., 1998). The disease affected all foliar plant parts, particularly on the panicles, and so was then termed 'panicle disease' or 'panicle blight'. The characteristic symptoms of the

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disease were blighting and necrosis of panicle, resulting in reduced flowering and reduced-to-absent fruit set, besides this, disease symptoms comprise necrosis of flower buds and failure to open, rapid expansion of "necrotic lesions" on buds and leaves. Typical bacterial lesion initiates as small water soaked spots which may join together and lead towards the raised lesions. Fluorescent pseudomonad was time after time isolated from the diseased panicles and identified as *Pseudomonas syringae* through LOPAT tests (Lelliot *et al*., 1966). Cazorla *et al*. (1998) and Torta *et al*. (2003) performed the Koch's postulates for the test bacterium and the etiology of *Pseudomonas syringae* pv. *Syringae* has been established. The pathogen was found in symptom appearance, particularly in cooler climates and it was noticed that the outbreaks of the diseases solely depends on winter season and spring season temperature whereas many other factors contribute for the disease development (Cazorla *et al*., 1998). It has also been noticed that dew factor has been found necessary for perpetuation of disease to peripheral buds, inflorescence and leaves while wind speed strongly promotes the disease through micro-injuries. The pathogen not only destroys the apical buds, inflorescence but also damage the leaves resultantly reducing the flowers and fruits set on mango tree. The apical bud necrosis disease manifestation was also noticed in the mango orchards of the Punjab, Pakistan and the current research was also aimed to isolate and identify the pathogenic bacterium based on the visual symptoms specifically on the apical buds and on inflorescence and to determine its incidence on some selective locations in the mango orchards because this disease was not reported from Pakistan before these investigations yet it needs more work to be done on this core issue of mango production.

MATERIAL AND METHODS

Study site: Current study was carried out at Department of Plant Pathology, Bahauddin Zakariya University, Multan (30.2639° N, 71.5101° E) and Mango Research Institute, Multan (30.1817° N, 71.4240° E 122 m altitude from sea level) in the mango zone of Punjab Province.

Bacterial isolation: The mango orchards at different regions of Multan and the experimental orchard of Mango Research Institute, Multan were evaluated for the occurrence of bacterial apical necrosis of mango on the basis of visual observations. Disease samples from the infected mango trees were collected, kept into icebox to avoid heat stress and transit injury and brought to the laboratory. Diseased samples were cut into 3-5 mm long pieces and surface disinfested in 5% ethanol for 1 minute, then washed thrice with distill water and placed in autoclaved (9cm) (Pyrex-Germany) Petri-dishes having autoclaved Nutrient Agar (NA) and incubated at 28 ± 1°C and observed daily for the bacterial colonies development (Dye *et al*., 1974).

Identification and preservation of bacteria: Fluorescent, white, circular and viscous colonies of the bacterium (*Pseudomonas syringae* pv. *syringae*) which developed on NA plates were pure cultured by streaking on King's B Medium and grown at 27 ± 1°C (Cazorla *et al*., 2005). The pure culture was preserved in 40% glycerol solution in autoclaved distilled water for the recovery of 16S rDNA in vials at -20°C.

Biochemical characterization

Gram staining: A smear of bacterial culture was made by spreading it uniformly on glass slide which was dried and fixed by passing it over a flame thrice. Then smear was stained with crystal violet or primary stain (having mixture of solution A containing crystal violet (90%)=2.0gm and Ethyl alcohol (95%)=20.0ml while solution B containing ammonium oxalate=0.8gm and distilled H_2O=80.0ml) and left for 30 seconds, after which it was washed for few seconds with distilled water and excess water was drained. Then diluted iodine (having Iodine=1.0gm, Potassium iodide=2.0gm and distilled H_2O=300.0ml) was applied for 1 minute, then washed with water and dried. Then decolorized with 95% ethyl-alcohol (having Ethyl alcohol (100%) =95ml and Distilled H_2O = 5ml) for 30 seconds, rinsed with water and air dried. Then counter stained with Safranine solution 25% (Safranine 10ml) in 95% ethyl alcohol (90ml) for 30 seconds, rinsed with water, dried and finally observed under microscope (Schaad *et al*., 2001).

KOH test: KOH test was performed in 3% KOH solution, a loopful of bacterial mass was smeared on glass slide in potassium hydroxide solution and smear was raised with inoculating needle to observe the reaction and counter signing the gram staining results (Ryu, 1940).

Hypersensitivity reaction: The pathogenic nature of the bacterium was studied on *Nicotiana tobaccum* (Tobacco) plants for hyper-sensitivity reaction as mentioned by Klement and Goodman (1967).

Disease incidence: The orchards were surveyed and divided into 4 blocks at three different locations viz., (Location-I: MRI, Multan), (Location-II: MRI, Multan),

(Location-III: BZU, Multan) to obtain the true picture of the prevailing bacterial apical necrosis disease. In each block twelve trees were randomly selected by adopting "Z" method irrespective of the variety in each block. The disease incidence was calculated by the following formula:

$$\text{Disease incidence (\%)} = \frac{\text{Number of diseased twigs/ inflorescence}}{\text{Total number of twigs/ inflorescence observed}} \times 100$$

Statistical analysis: Datasets of disease incidence of all the four blocks were averaged and subjected to statistical analysis using the analysis of variance (ANOVA) comparing by Duncan New Multiple Range test (DMRT) at ($P \leq 0.05$) via using SAS (Statistical Analysis System, version 8.1).

RESULTS

Pathogen isolation, identification and biochemical characterization: The pathogen was successfully isolated on nutrient agar medium which is a general purpose medium for the isolation of most of the bacterial species. Besides this bacterium was also purified on King's B Medium by streaking, all the plated bits showed maximum recovery of the bacterial masses on nutrient agar medium with bright color flattened colonies. Red and pink colors were observed after gram staining procedure when counterstained with safranin under the microscope at 100X magnification with the help of cedar oil; which confirmed that bacterial cultures were gram negative i.e., phytopathogenic, bacteria have the ability to cause disease in mango trees as bacterial apical necrosis. Besides this, KOH test also confirmed the results of the gram staining by producing mucoid thread like structure i.e., bacterium is gram negative. The pathogen proved hypersensitive reaction positive on *Nicotiana tobaccum* leaves that could only be stimulated by plant pathogenic bacterium due to tissue collapse and absolute necrosis during 24-to-48 hrs (Table 1, Figure 1).

Figure 1. Bacterial apical necrosis disease symptoms: A: twig blight, B: Inflorescence blight, C & D: Bud necrosis.

Table 1. Biochemical and diagnostic test for Pseudomonas syringae pv. syringae, potential agent of bacterial apical necrosis.

Pathogen	Biochemical test		Diagnostic test
Pseudomonas syringae pv. *syringae*	Gram staining	*KOH	Hypersensitivity reaction
	+	+	+

* Potassium hydroxide test.

Disease incidence: Survey results showed that all the tress in each locations of the experiment depicted the presence of the apical necrosis disease with the most obvious symptoms on young twigs and inflorescence blight and necrosis of vegetative and flower buds and bud failure. Maximum disease incidence was recorded on location-2 with 46.66% while minimum disease incidence was recorded on location-3 with 13.33%. Location-1 ranges from 25 to 41.66% disease incidence while location-2 ranges from 31.33 to 46.66% whereas location-3 ranges from 13.33 to 43.33% disease incidence (Figure 4, 5, 6).

Figure 2.*Pseudomonas syringae* pv. syringae colonies on nutrient agar.

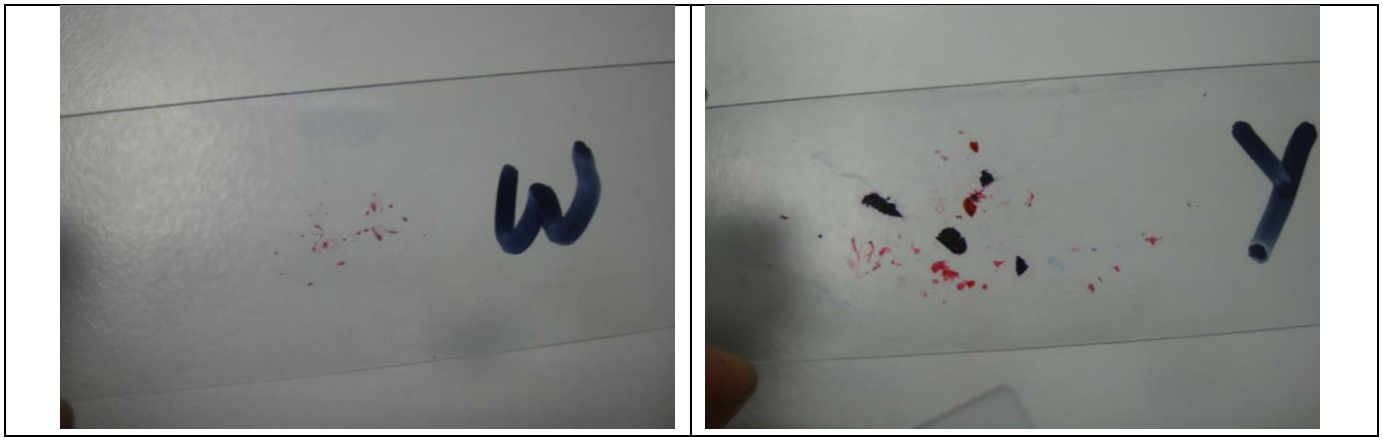

Figure 3. A: White color bacterial colonies smear showing red color and B: whitish yellow color smear showing pink in response of gram staining reaction on glass slide.

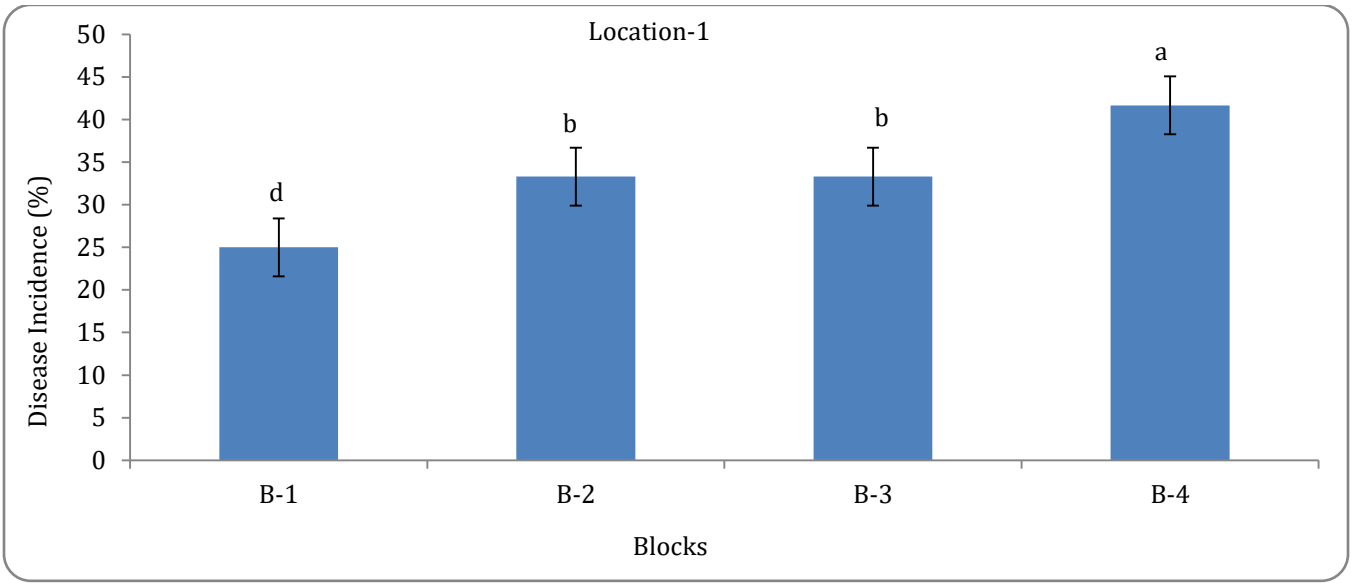

Figure 4. Location-1, representing the picture of bacterial apical necrosis disease in four blocks .

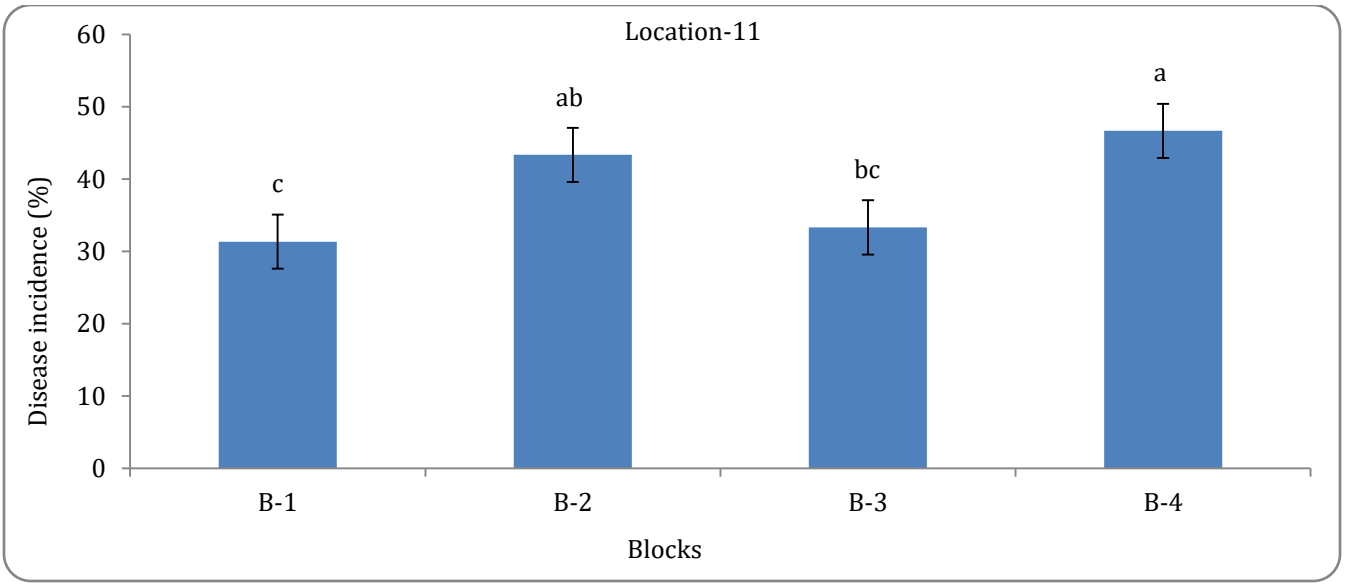

Figure 5. Location-2, representing the picture of bacterial apical necrosis disease in four blocks.

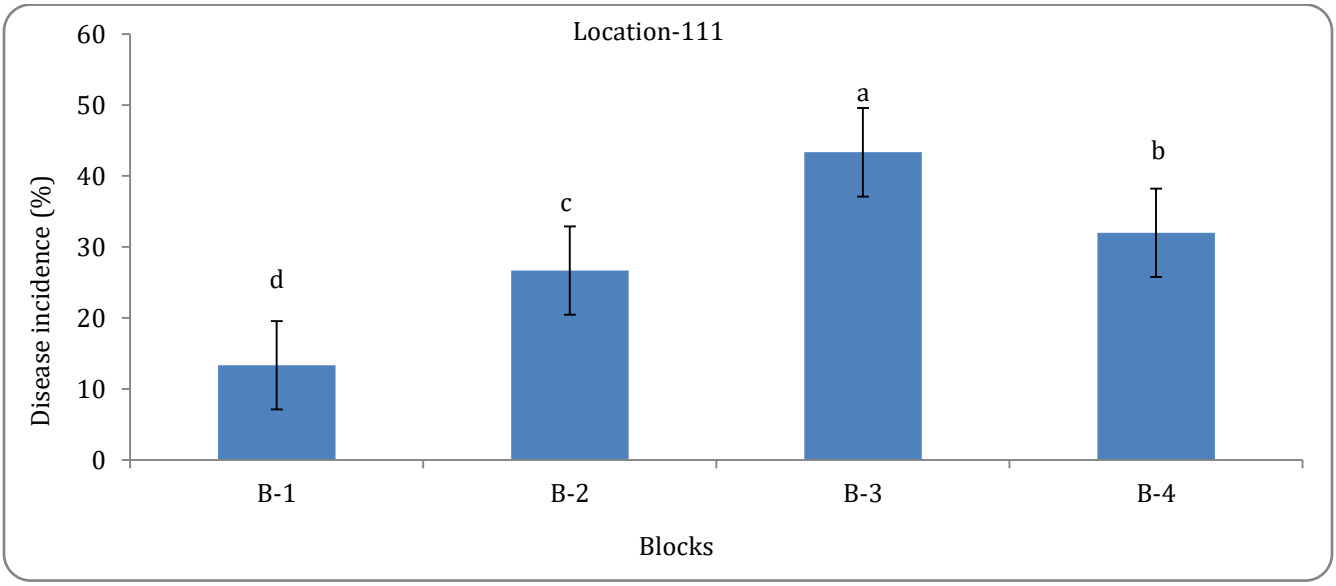

Figure 6. Location-3, representing the picture of bacterial apical necrosis disease in four blocks.

DISCUSSION

Apical bud necrosis, twig blight, apical necrosis, panicle blight, panicle disease, bacterial black blight etc are the same name of the bacterial apical necrosis disease. The disease has been identified and established by the Koch's postulates in Australia, Israel and Purtagal where mango cultivation is hampered by this damaging pathosystem. The disease is characterized by some typical symptoms on new buds, new twigs, and inflorescence and leaves wherein diagnostic symptoms include vegetative and flower buds necrosis and failure to open, a rapid expansion of necrotic lesions on buds and leaves. The symptoms on leaves start as angular, intervenial, water soaked spots which coalesce and become black with the passage of time in cool seasons when bacterial population multiplies rapidly (Torta *et al*., 2003). *P. syringae* pv. *syringae* has been established as the pathogen of this disease because *P. syringae* populations exist within diverse microbial communities on nearly all of terrestrial plant species. *P. syringae* pv. *syringae* possessed a wider range than other pathovars, and might be present in epiphytic or pathogenic associations within the population (Hirano and uppar, 1990). As for as mango is concerned, this bacterium elicits a disease

known as bacterial apical necrosis, and is considered one of the major factors limiting mango fruit production in Southern Spain and Portugal (Cazorla *et al*., 1998). In our investigation, we found the same symptoms on the plants which are specifically affected by the bacterium in terms of blight and we also isolated the bacterium on nutrient agar and King's B medium with luxurious colonies. Our results are in line with Francisco *et al*., (1998) who described, isolated and characterized *Pseudomonas syringae* pv. *syringae* as causal agent of apical necrosis disease of mango in Spain by culturing diseased tissue and streaking on NA and KB medium. Similarly our findings are also counter signed by Galal *et al*., (2006) and Cazorla *et al*., (2005) who also isolated the bacterium from the blighted twigs and flowers and maintained the pathogen culture on NA and KB medium. NA is a general purpose medium for the culturing of wider range of bacteria including pseudomonas and proved a sound alternative if there is no KB medium available. Similar results were also obtained by Shenge *et al*., (2008) and Arrebola *et al*., (2003) who experimented streaking loopful of suspension of macerated tissues in few drops of sterile distilled water on KB medium plates and incubated for 3 days at 25–28°C to obtain luxurious colonies of *P. syringae* pv. *syringae.* Our facts are in parallel with Young (2008) who documented *Pseudomonas syringae* pv. *syringae* as causal agent of bacterial necrosis disease of mango as the same type of symptoms were observed in their findings in mango orchards. Although the infection dynamics of the *P. syringae* pv. *syringae* has also been established on many other crops like tomato but its role in mango disease is more important because due to the apical bud necrosis or bacterial apical necrosis the number of flowers becomes lessen in number and fruit set may become delayed which result a significant yield loss. In our experiment we assessed the disease incidence of the bacterial apical necrosis in some selected areas of Multan and found a significant prevalence of the disease in the mango orchards of the Multan. The increasing incidence of the diseases demands a timely management strategy to avoid severe losses of the disease in the future scenarios. In our observation disease was more prominent in cool days when temperature becomes less than 15°C after rain fall. We observe the appearance of disease symptoms after the rainfall more rapidly as compared to the sunny days. Disease incidence was observed more on mango trees possessing the dense canopy as compared to the pruned trees. With the best of our knowledge, these findings possess the first and foremost information on bacterial apical necrosis disease of mango in Multan, Punjab, Pakistan. Yet, it is the dire need of time to study the genetic diversity of the pseudomonas population in mango orchards to acquaint the occurring problems. Many aspects of this disease needs attention of the scientist to explore, probably the epidemiological studies and molecular characterization of the pathogen will initiate the new avenues to find more reliable solutions of this disease. Well, the changing pattern of the climatic conditions in the world and specifically in Mango zone of Pakistan might also be the driving force for disease the disease to establish in this area.

CONCLUSION

From our investigations, we conclude that increasing incidence of the disease in mango orchards requires management strategies to control this problem before it is established. Epidemiological factors might play a significant role in the development of the disease.

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