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CHARACTERIZATION OF *ERWINIA AMYLOVORA* CAUSING TWIG BLIGHT OF LOQUAT TREE IN LOQUAT GROWING AREAS OF PUNJAB AND KPK, PAKISTAN

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ABSTRACT

Twig blight of loquat (*Eriobotrya japonica* Lindl.), caused by the gram-negative enterobacterium Erwinia amylovora, is a significant constraint to loquat production worldwide. Recent surveys in loquat-growing areas of Punjab and KPK, Pakistan—specifically in Chakwal (Kalar Kahar, Choa Saidan Shah), Rawalpindi (Taxila, Wah), Murree (Tret and Chattar), and Haripur (Haripur, Khanpur, and Sara i Saleh)—revealed the high prevalence of this disease. Twig blight compromises the health and vigor of loquat trees, often leading to their death, which discourages farmers from cultivating loquat commercially. Consequently, the area under loquat cultivation and its production have both been declining. Despite the severity of the issue, no systematic studies had been conducted to address this problem until now. A survey was carried out over two consecutive years (2016 and 2017) in 10 loquat-growing locations in Punjab and KPK (three orchards per location). The incidence of twig blight was higher in 2017 compared to 2016. The highest incidence was recorded in Taxila, with 69.74% in 2016 and 78.39% in 2017, while the lowest was in Kalar Kahar (20.86%). The incidence in Kalar Kahar showed a slight increase of less than 1% from the previous year, likely due to effective control measures implemented to limit the spread of the disease. The pathogen *E. amylovora* was isolated from diseased samples. Out of 102 bacterial isolates, 92 strains were gram-negative, and 72 tested positive in various biochemical assays (Levan, lipase, KOVAC oxidase, glucose fermentation, catalase oxidase, growth at 39°C, production of fluorescent pigment, and pectolytic activity tests). Three highly virulent isolates were identified through pathogenicity tests and confirmed by biochemical and molecular indicators. Molecular tagging of two chromosomal genes (amsB [MN902191] and Pst-glms [MN915084]) and one plasmid gene (pAE29 [MN902192]) was performed, confirming the identity of the isolates as *E. amylovora*. This study represents the first report of *E. amylovora* infecting loquat in Pakistan.

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INTRODUCTION

Loquat (*Eriobotrya japonica* Lindl), a species of evergreen flowering plants. It is a subtropical fruit tree of *Rosaceae* family indigenous to central china in the subcontinent of Asia (Hussain *et al.*, 2006). Records of loquat from China discerned over 2000 years ago. Then it was introduced into Japan and has been cultivated there for over 1000 years. According to the historical background of loquat, originally it belongs to southwestern China during prehistoric times and its cultivation was shifted to Japan as early as 1180. Presently, it is being cultivated worldwide at moderate

altitude including China, Pakistan, India Madagascar, Japan, Africa, Europe, United States, Australia and Brazil (Khan, 2003).

Khan (2003) and Hussain *et al.* (2006) stated that loquat is an important fruit crop of Pakistan. It is specifically cultivated in areas where abundant amount of canal and spring water is available. Areas including Kasur, Sargodha, Kalar Kahar, Choa Saidan Shah, Chattar, Tret, Hassan Abdal, Wah, Harripur and Mardan. In Punjab it is grown in Kalar Kahar and Choa Saidan Shah (Hussain *et al.*, 2006). It is the first fruit of the year developed during winter and ripens in early April. That's why it is very popular and gives good return. Pakistan tends to produce fine quality of fruit for export purpose; still it is mainly consumed in local markets. Besides being juicy and delicious, it is extremely high in nutrition. The main constituents of loquat are vitamins (A, B and C), minerals (phosphorus & calcium) and carbohydrates (Karadeniz and Şenyurt, 2006). Leaf extract of loquat believed to have great medicinal value (Wee and Keng, 1992). Leaves are also used in medicines to cure skin diseases and alleviate inflammation, cough and pain (Nishioka *et al.*, 2002).

Loquat fruit has been found suffering from many diseases. Many pathogens have been reported to attack loquat fruit worldwide like *Fusicladium eriobotryae* (Sánchez-Torres *et al.*, 2009; Caballero and Fernández-Zamudio, 2004), *Colletotrichum* sp. (Wee and Keng, 1992; Karadeniz and Şenyurt, 2006), *Diplodia* sp. (Persley *et al.*, 2010), *Erwinia amylovora* (Starr *et al.*, 1951; Momol and Aldwinckle, 2000) and *Alternaria* sp. (Batta, 2003), *Pseudomonas eriobotryae* (Lai *et al*., 1972). Among the above mentioned diseases, twig blight, a major pathological constraint pertaining to loquat fruit production in many areas of world (Kabeil *et al.*, 2010), is caused by *Erwinia amylovora,* a gram-negative bacterium (Piqué *et al.*, 2015). It is reported that there are more than 39 genera comprising 180 species amongst *the Rosaceae* family. Its host range is restricted to the members subfamily of family *Rosaceae* that is affected by *Erwinia amylovora* i.e. *Pyrus* sp., *Cydonia* sp., *Malus* sp., *Cotoneaster* sp., *Crataegus* sp., *Eriobotrya japonica*., *Sorbus* sp. and *Pyracantha* sp. (Starr *et al.*, 1951). Burrill described *E. amylovora* as the first plant pathogenic bacterium in 1893. *Erwinia amylovoran*, a G-ve bacterium belongs to the family *Enterobacteriacea*. It is the first phyto-pathogenic bacterium described ever (Vrancken *et al.*, 2013). Initially the disease was observed in North America during the 18th century, later it spread to other parts of the world. This pathological constraint have been reported in more than 50 countries up till now, however, there are some regions exist that are devoid of this disease (Zhao *et al.*, 2019).

Concerning severity in fruit trees, twig blight has no competitiveness to any other diseases. It's clearly mentioned in different studies that the infected trees can expire (even at the young stage) during one season and expectedly the older orchards within a few years. In case of high infestation in diseased orchards 20 to 50%, in some cases even up to 100% of trees may be affected (Shemshura *et al.*, 2020). Tancos *et al.* (2017) mentioned that the growers of apple orchards also have experienced up to 50% tree losses even in freshly planted orchard in New York, all due to unexpected and sudden twig blight subsequently followed by blossom blight. Such losses amplified when the orchards were converted into high density plantings. During recent surveys in loquat growing areas of Pakistan, the disease was found most prevalent. This disease destroys the tree in fullest health and vigor and is responsible for an ultimate death of whole tree that is averting the farmer to grow loquat for commercial purpose. Due to this pathological constraint, the area is decreasing and so the production. No systematic study has been conducted so far to address the problem. Hence, the present study is planned with the objective of characterization of isolates associated with the twig blight of loquat tree.

MATERIALS AND METHODS

A symptomatic survey of loquat orchards was conducted in 4 loquat growing districts of Punjab and KPK viz. Chakwal (Kalar Kahar, Choa Saidan Shah), Rawalpindi (Taxila, Wah), Murree (Tret and Chattar) and Haripur (Haripur, Khanpur and Sara i saleh) in the months of April (2016) and April (2017). Three orchards were selected from 10 locations and from each orchard all trees were observed individually for the visible symptoms of twig blight disease. The distance between each orchard was approximately 5-7 kms. 5 Five representative samples were randomly collected from each orchard. Prior to candidature declaring the symptomatic tree infected by twig blight disease, the twigs were inspected for any breakage or injury from their point of attachment. The damaged twigs generally exhibit typical symptoms of blighted twigs. Therefore, only the intact branches were selected for sample

collection. While selecting the twigs for sample collection it was also considered that these must have borne blighted fruits from the previous fruiting season. Suspected twig samples were collected from loquat trees on the basis of preferred symptoms. A total of 30 orchards from 10 locations of 4 loquat growing districts of Punjab and KPK were surveyed. Over all 165 twig samples were collected from these locations. The infected twigs were removed for further investigation and labeled (date, orchard number and location) and transferred to the Department of Plant Pathology Laboratory at Pir Mehr Ali Shah Arid Agriculture University, RWP.

Isolation and Purification

In order to perform isolations Nutrient agar (20g NA in 1000ml distilled H_2O) was prepared and sterilized at 121°C for 15 min. Infected twig samples were cut into pieces and soaked in 10% Clorox for two minutes, then rinsed three times in sterilized distilled water (one minute each time). The pieces were dried by placing them on sterilized filter paper to remove extra water. The dried pieces were placed over the media in already poured media plates in such a way that the infected portion touched the media surface. Maximum four pieces were oriented 1 cm away from the peripheral area of a Petri plate. The plates were parafilmed followed the incubation at 25°C until visible growth was observed. After 24-48 hours the bacterial colonies emerged around the pieces of infected twigs. The suspected pathogen *Erwinia amylovora* comprises creamy white colonies, therefore, streak plate technique was employed by four directional streaking on an agar plate from the resulting colonies exhibited creamy white color to produce isolated colonies of an organism. The inoculated plates were parafilmed and incubated again in an incubator. After incubation of 24-48 hours, purified cultures were obtained and were subjected to further assessment.

Determination of Gram Negative Bacteria

Since, the suspected pathogen *Erwinia amylovora,* belongs to the phylum Proteobacteria (Eastgate, 2000), therefore considered as a Gram-negative bacterium (Vanneste, 2000; Vrancken *et al.*, 2013). Hence in order to separate Gram-negative bacteria from the bacterial genetic pool of isolates, different tests viz HR, Gram staining and KOH test were performed.

Hypersensitive response (HR) is an imperative and definitive examination test to distinguish phytopathogenic bacteria from saprophytic microorganisms. HR-inducing ability test was confirmed on tobacco leaves (cultivar *Nicotiana tabacum* L). The procedure followed for the inoculation of bacteria was described by Umesha *et al.* (2008). The cell suspensions from 24 hours old bacterial isolates were prepared in sterile distilled water and inoculum was diffused into the intercostal area of young and fully expanded leaves of tobacco with sterile needles. Sterilized water was used as a control. The plants were covered with polythene bags and left for 18-24 hours for symptoms appearance.

Gram staining test also helps to separate Gram +ve and Gram -ve bacterium. Additionally, it also helps in examining the shape, arrangement and staining reaction of bacterial isolates. Gram staining was performed following the method explained by Islam *et al.* (2017). A 24 hours old culture was obtained and smeared on a slide and fixed by heat with care. One drop of crystal violet was placed on a slide, smeared and held it for one minute and then rinsed with distilled water. Extra water was removed from the slide. Then one drop of gram's iodine was placed on, smeared for 30-60 seconds and rinsed with distilled water. Then decolorizing reagent (we used 70% ethanol) was added on the sample for 5-10 seconds. If the sample was Gram-negative the purple shade was completely removed. In the end, safranin was added for 30 seconds and rinsed using distilled water and air dried for a few minutes. The slide was then observed under 100X microscope (by adding a drop of immersion oil over slide) to examine the cell characteristics of bacteria.

Compared to other methods, KOH is more rapid and precise. A drop of 3% KOH was placed on a slide. 24 hours old bacterial cells were removed aseptically from cultures, with the help of a sterilized toothpick. The cells were slightly smeared on a glass slide and agitated rapidly in a circle. In case of Gram-negative strains, the suspension became viscous, and a loop appeared while lifting the toothpick. However, when the bacteria was gram-positive the cells dispersed into the drop and did not show the above mentioned reaction.

Biochemical Studies

After confirming their G-ive nature, isolates in this study were further analyzed by a series of biochemical tests for the conformations of *Erwinia amylovora viz*: Levan Production Test (with 7 % sucrose) (Schaad, 1980), Lipase Activity Test, Pectolytic Activity Test (Fahy and Parsley, 1983), Kovac Oxidase Test (Schaad, 1980),

Catalase Oxidase Test (Schaad, 1980), Glucose Fermentation Test, Production of Fluorescent Pigment.

Pathogenicity Test

The whole experiment was conducted in an orchard located in Wah during 2017-2018. The most virulent strain was confirmed by pathogenicity test. For that purpose, a total of 57 isolates were recovered after confirmation through different cultural (colony color and shape) and biochemical characteristics (Levan and lipase production, change in color of KOVAC reagent and bubble formation in hydrogen per oxide). Each isolate was inoculated on healthy young loquat plant followed the protocol by Bell *et al.* (2005) and on the young detached leaves by following the method of Mitrev and Kostadinovska (2016) with three replications. For young plants fresh-cell inocula was harvested from a 24 hrs old, cultured plates, suspended in liquid and cell density was adjusted at 600 nm using a spectrophotometer to approximate of 1×10^7 CFU/ml. The plants were sprayed during the blossom stage. The inoculation was done during the day when the air frequency is less. Petal of flowers were trimmed from the tip and dipped into the inoculum. The blossoms were then covered with polythene bags. For the control treatment liquid broth is used alone. The blossoms were monitored after every 7 days. In total, 171 stems were inoculated with 57 isolates of bacterial isolates.

After the close examinations, symptom categories were established. A rating scale comprising numerical scores, increased with increasing significance of the symptom. The scoring was done according to 0-6 scale in order to evaluate plant disease development as explained by Bell *et al.* (2005) with a very slight amendments shown in table 1.

A % disease severity index (DSI) of pathogenicity was estimated from the symptom score values and their frequency of occurrence by using formula by Chiang *et al.* (2017).

$$\text{DSI (\%)} = \frac{[\text{Sum (class frequency} \times \text{Score of rating class)}]}{[(\text{Total number of plants}) \times (\text{Maximal disease index})]} \times 100$$

In the case of detached leaves, the entire surface of the leaves were sterilized with 70% ethanol. Media was poured in a Petri plate and after solidified the pathogen was streaked over its surface. Injuries were made mechanically on leaves and placed on the poured plates. The plates were wrapped with parafilm and incubated. After every 24hrs the plates were observed for symptom development. From 3-7 days of inoculation, leaves were assessed for leaf blight symptoms based on disease scale developed by Rahayu (2014) from 0 to 4, where 0= no infection, 1= 1-25% of leaf area blighted, 2 = 26-50%, 3 = 51-75%, 4 = 76-100% of leaf area blighted. A % disease severity index (DSI) of pathogenicity was calculated from the increasing symptom score values and their frequency of occurrence by using formula by Chiang *et al.* (2017).

PCR Optimization of Pathogenic Isolates

DNA extraction of isolated pathogen was done by using direct colony PCR (Espinosa *et al.*, 2013). The colony from 24 to 48 hrs old culture was slightly touched by a sterilized toothpick and added in an Eppendorf tube containing TE buffer (1.21g Tris 10mM & 0.29g EDTA added in 1L distilled water). The water in the water bath was preheated at 90°C. These tubes were placed in pre heated water for 15-30 min. Lastly, centrifugation was done on the sample tubes at 13000 rpm for 15 minutes. Supernatant was taken as ready to use DNA template for PCR analysis. The purified extracted DNA was then assessed by electrophoresis. DNA extracts from the collected samples were then subjected to PCR optimization. Molecular characterization of bacteria was done by using specific primers of *Erwinia amylovora*. PCR reaction and conditions (Table 2) were optimized for the successful amplifications of *Erwinia amylovora*.

Confirmation of PCR Products

The electrophoresis of PCR generated fragments was carried out in 1x tris-borate buffer. The buffer was boiled for one minute to completely dissolve the agarose. Then ethidium bromide (2µl) was added to the lukewarm gel solution before pouring into gel try. After the gel was cooled, the tray was fixed into the electrophoresis apparatus, with the wells near the negative electrode. The electrophoresis buffer was poured until it gets 5mm above the gel. 2 µl of loading dye and 8 µl of DNA was loaded in each well. Agarose gel electrophoresis (80 volts for 45 min) was carried out and then the gel was transferred to the gel documentation system and photographed. Product size was estimated by comparison with 1500 bp standard ladder. Amplified products were sequenced using the same primers which were used for PCR. The obtained sequences were aligned using ClustalW2, analyzed with the basic local alignment search tool (BLAST) program of National Center for Biotechnology Information

(NCBI) and compared with sequences of those already deposited in GenBank.

Phylogenetic Analysis

Phylogenetic analysis was done by constructing Phylogram with MEGA-X using the maximum likelihood method.

Table 1. Disease rating scale (0-5) for the disease development in plant.

Disease Score	Symptoms Details
0	No evidence of twig blight
1	Blossom blight
2	Blossom blight and discoloration of a few (1-3) leaves.
3	Browning of (>3) leaves along with browning of twigs
4	Blackening of twig infected and considerable dieback
5	Shrunken and cracked twig with a few or no leaves

Table2. Molecular characterization of virulent isolates.

Primer name	Sequence	Annealing temperature (°C)
pstS––glmS	CCGAAGAACGATTGCACTAC CGGTTAGTTAGCGCAGTCTC	52
amsB	GCTACCAGCAGGGTGAG TCATCACGATGGTGTAG	49
pAE29	TTCACGGCTTCGCAGAT ACCCGCCAGGATAGTCGCATA	52

RESULTS AND DISCUSSION

Disease Incidence of Twig Blight from the Growing Seasons of 2016-17

The twig blight of loquat was found prevalent in all orchards. No orchard was found free from the disease. The incidence of twig blight was relatively higher in 2017 than the previous year (Figure 1). During both study years (2016 & 2017) the maximum twig blight incidence i.e. 69.74% and 78.39% was recorded in Taxila respectively. It is also evident from the figure 2 that the maximum increase in incidence of twig blight (8.65%) was observed in Taxila with the lapse of just one year. The incidence was, however, approximately same in Kalar Kahar and there was a very slight increase of twig blight i.e. less than 1% compared with the previous growing season (Figure 2), which may be attributed to some pragmatic control measures adapted to limit the spread of twig blight.

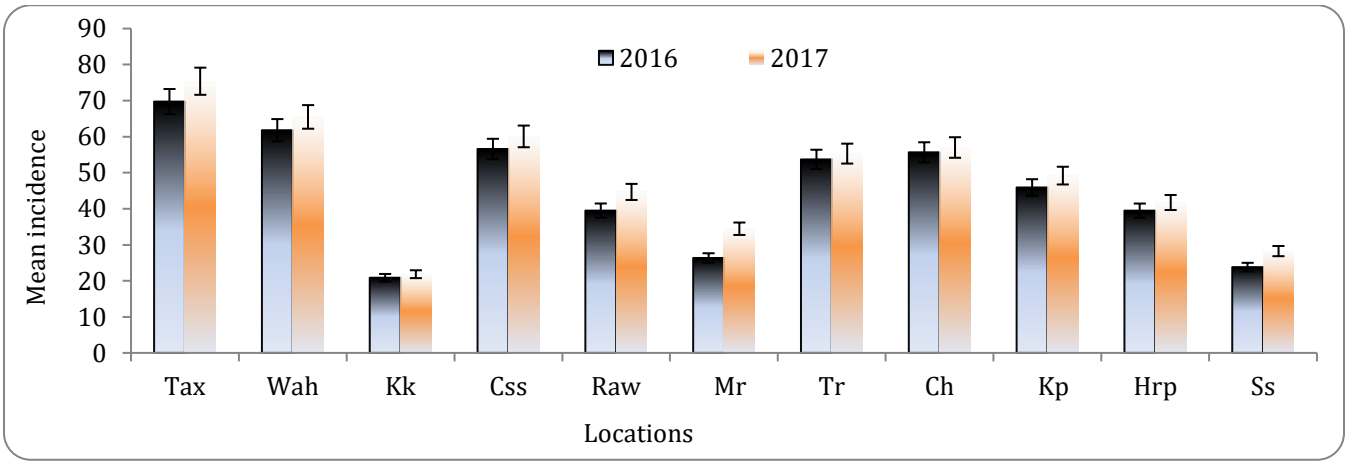

Figure 1. Percent disease incidence of twig blight from the growing seasons of 2016 & 2017.
(Tax; Taxila, Wah: Wah, Kk: Kalar Kahar, Css: Choa saidan shah, Raw: Rawalpindi, Mr: Murree, Tr: Tret, Ch: Chattar, Kp: Khan pur, Hrp: Hari pur, Ss: Sarai saleh.)

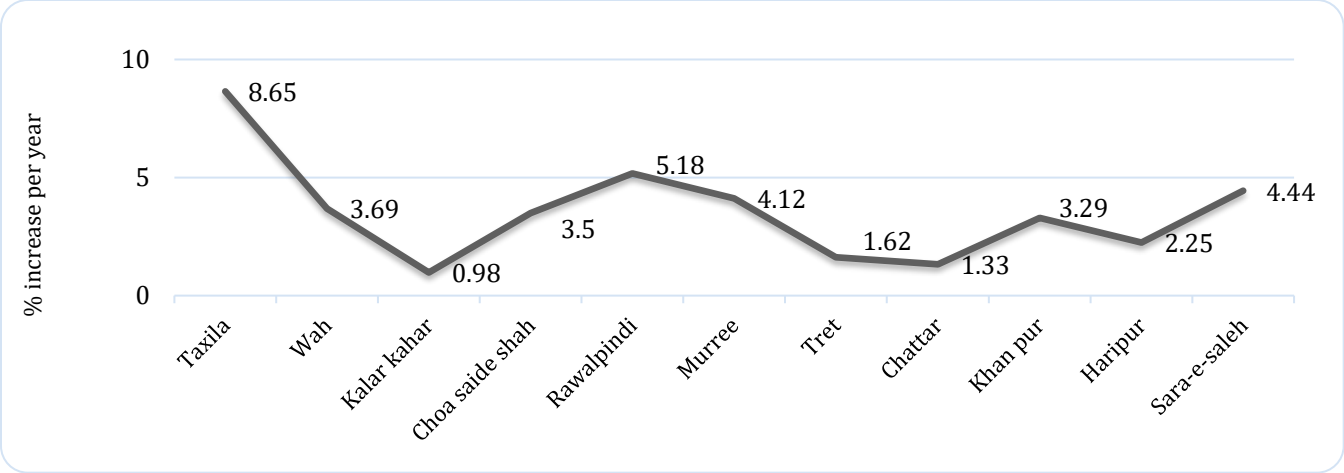

Figure 2. Percent increase of TBL disease incidence per year.

The twig blight of pome fruits is an intensively studied bacterial disease in apples and pears, however, this disease was least studied against other pome fruits including loquat. The disease is continuously spreading and making it a serious concern in loquat growing areas of Punjab and KPK. The spread may be attributed to the lack of cultural practices (sanitation and pruning) leading to a very thick, messy and overcrowded plantation that reduces the sunlight which in turn increases the humidity.

All these factors favor the establishment of a new infection throughout the season that finally leads to the loss of the orchard at the end of 2017. Johnson (2000) also mentioned that the epiphytic growth of *Erwinia amylovora* on stigma along with the flower to flower movement of the pathogen by pollinating insects are the other essential processes that influence the blossom blight incidence of. *Erwinia amylovora*. According to him the pathogen can be able to survive on other healthy plant surfaces e.g. leaves and branches, for a limited period of time (weeks). The results are also in accordance with Schoofs *et al.* (2020). He observed that the harshness of the disease is preferred by specific weather circumstances such as high humidity and temperature especially during the vegetative growth.

Isolation and Purification

A total of 92 bacterial isolates with typical colony characteristics i.e doomed shape off -white colonies were purified from the 165 collected samples. High levels of homogeneity among isolates were observed where colonies were typically white, creamy white and domed (Figure 3). The isolates were tagged/named according to the area of sampling, and further examined for Gram negativity by employing different tests.

Figure 3. Cultural characteristics (doomed shape, off white colonies) typical to *Erwinia amylovora* (Tax2a strain).

Determination of Gram Negative Bacteria

As the required bacteria is of gram negative nature, the purified isolates with colony characteristics typical of *E. amylovora* were tested for Hypersensitivity test, gram staining and KOH test to separate G –ve from G +ve bacterial isolates. Among 92 isolates, 57 were found gram negative. They exhibited localized cell death in the intercostal region of tobacco leaves (Figure 4a) within 24 -72 hrs after the infiltration of the bacterial cells into tobacco leaves @ 1×10^6 cfus ml^-, whereas no HR was observed in remaining 35 isolates thus identified as G+ve.

During gram staining test, all of the 57 (HR positive) isolates retained pink color (Figure 4b), and during KOH test the loop was formed as the continued agitation increased the viscosity of the liquids (Figure 4c) because the DNA obtained by the lysed cell of Gram-negative bacteria lifted from the slide surface on the toothpick, created a loop on slides when it is drawn up slowly. Both gram staining and KOH tests further confirmed their gram negative nature. However, remaining 35 isolates retained blue color during gram staining test and no viscosity was detected in the KOH solution, thus considered as Gram-positive bacteria.

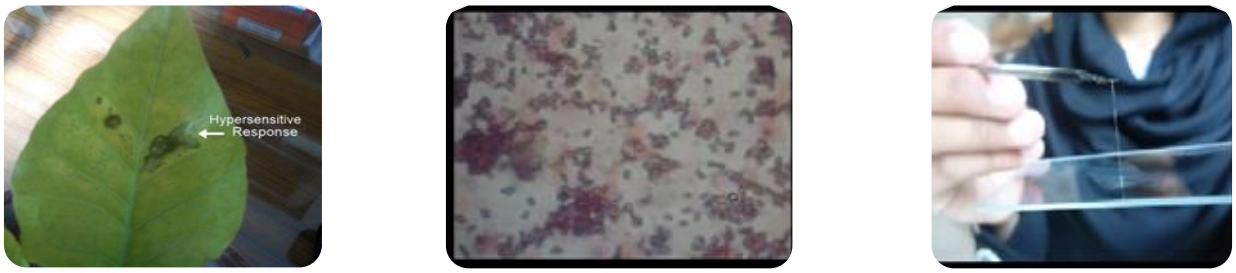

Figure 4. Determination of gram negative bacteria from the genetic pool of different isolates .(a) HR test (b) Gram staining test (c) Loop test.

To identify G-ve bacterium we mostly rely on the Gram staining, kOH and HR as preliminary tests. The reason for the use of these three tests simultaneously is that if any of the test misinterpreted, the organism may be misidentified, which would result in misdiagnosis. So, in this study the isolates that fulfilled the requirement of above mentioned tests were taken for further experimentation.

Most G-ve phyto-pathogenic bacteria give hypersensitive response in tobacco (*Nicotiana tobaccum* L.) whereas, G+ve bacteria in four o'clock (*Mirabilis jalapa* L.) (Umesha *et al.*, 2008). The fabrication of a hypersensitivity response (HR) is an essential property of plants pathogenic bacteria and the virulence of this plant associated bacteria mostly depends on their capability to produce an HR on non-host plants. Among many factors compulsory for virulence, the HR induction is the most important one (Alfano and Collmer, 1997; Jakovljevic *et al.*, 2008; Yang *et al.*, 2020). Almost all phyto-pathogenic bacteria produce an HR reaction in leaf's mesophyll tissues. Whereas, this reaction does not induct by saprophytes, which makes HR a rapid and helpful test to differentiate phytopathogens from saprophytic micro-organism (Braun-Kiewnick, 2001).

Another very important method to distinguish G-ve from G+ve bacteria is Gram Staining. Gram stain was initially introduced in 1884 by Hans Christian Gram (Smith and Hussey, 2005). The cell wall of G+ve bacteria contains thick layers of peptidoglycan, comprises 90% of the cell wall. That is why the Gram-positive isolates stain purple. On the other hand, cell wall of G-ve bacteria consists of thin layer of peptidoglycan i.e. 10% of wall, as well as high lipid content. Misunderstanding of the Gram staining has directed to misdiagnosis of such infective diseases; (Noviello *et al.*, 2004). Whereas, in KOH test the liquid became viscous in 15-30 seconds due to lysis of Gram-negative bacterial cell occurred in the 3% KOH. In this study those isolates that retained pink color, formed loop and showed necrosis on tobacco leaves were selected for further study.

Biochemical Characterization

The g –ve isolates were characterized biochemically for further identification. Out of 57 isolates 36 isolates exhibited the biochemical characteristics typical for *Erwinia amylovora.* They showed positive Levan formation by producing circular, raised domed, smooth, mucoid and shiny colonies. In lipase test these isolates produced opaque zone and milky white dense precipitate around the bacterial colonies after 3-4 days of incubation. For pectolytic activity test rotting was extensive throughout

the inoculated potato plugs and bacterial oozing was evident that might be the indication for *E. amylovora* presence. In the case of KOVAC oxidase test change in color from white to purple was observed and *E. amylovora* is supposed to produce purple color when its colonies are rubbed on filter paper was dampened/sprinkled with 1-2 drops of KOVAC oxidase reagent. Moreover, when exposed to H_2O_2 these isolates survived the oxidative stress and produced bubbles in a tube containing H_2O_2, as they possess an enzyme which can break H_2O_2 into H_2O and O_2, and these liberated O_2 produced bubbles. In the case of glucose fermentation test the change in color of the basal medium from yellow to green was after 3-4 days of incubation. The change in color is due to the fact that the acid is produced in the tubes as a result. Lastly, non-fluorescent colonies are produced on KB medium when exposed to UV light. The results are shown in Figure 5**.**

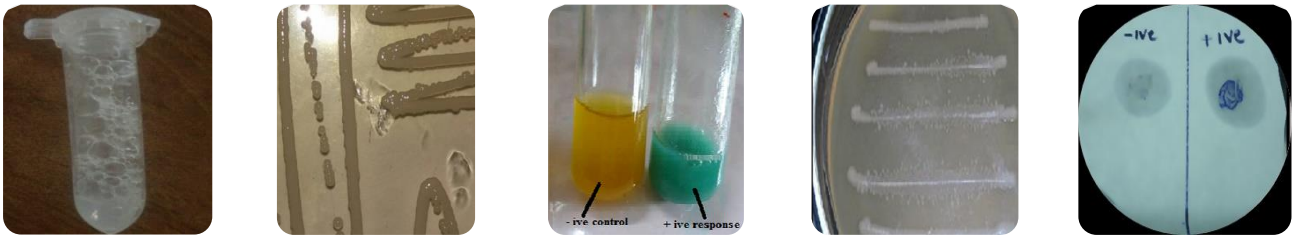

Figure 5. Biochemical characterization of pathogenic bacterial isolates. (a) H_2O_2 test. (b) Levan test. (c) Catalase oxidase test. (d) Lipase test. (e)KOVAC test.

All traits shown by biochemical tests revealed that all of the 36 isolates were typical to those of *Erwinia amylovora.* Furthermore, the results are also in agreement with the results of Ashmawy *et al.* (2015) and Shoeib *et al.* (2016). The production of Levan, intended to be one of the virulence factors of the bacterium. The Levan production is carried out by the help of Levan sucrose (Lsc) enzyme. Levan sucrose enzyme catalyzes the hydrolysis of sucrose yielding fructose and glucose in low sucrose concentrations. However, the high sucrose concentration encountered during flower infection, there occurred a trans-fructosylation in a ping pong mechanism, during this process a fructosyl moiety is transported by an enzyme-fructosyl intermediate to an acceptor, The acceptors can be either fructose or sucrose thus give rise to short chain FOS (fructo-oligosaccharides), or a long-chain Levan (Szwengiel *et al.*, 2007). Dewey *et al.* (1999) found a cloudy zone around the periphery of bacterial colonies during lipase test. He saw crystals of calcium oxalate in this cloudy zone upon microscopic examination indicative of lipolysis that had taken place.

Pathogenicity Test

Pathogenicity test showed that all bacterial isolates were able to infect the plants with an erratic pathogenic ability as the degree of aggressiveness varied among the isolates. The symptoms of blossom blight began to appear after 7 days of inoculation. Then progressed towards shoots that become blighted after 15-20 days of inoculation. After 21-35 days of inoculation, from shoots the pathogen moved towards leaves and caused browning as they may burned by fire. And finally the shoots appeared shrunken and cankered. Results are shown in Figure 6 A and B.

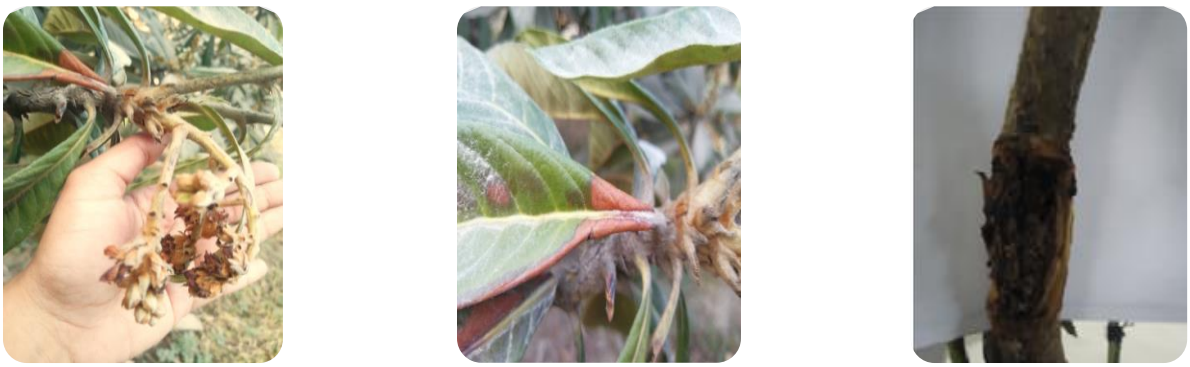

Figure 6 (A). Pathogenicity test performed for the validation of pathogen. (a-c) Pathogenicity test *in planta.*

Among 36 isolates 3 isolates manifested highly virulent response as they followed the scale from 0-6, 19 isolates were moderately virulent (followed the score 0-4) and 14 were less virulent (followed the score 0-2) and the isolates viz Tax2a, Tax11b Wah13c followed the score of 0-6 had the highest severity index of 100%. On the contrary, in uninoculated plants no symptoms appeared. The progress of the disease is shown in Figure 6 A and B.

In the case of artificially inoculated detached leaves similar isolates Tax2a, Tax11b Wah13c performed well and showed 100% severity index. All bacterial isolates induced typical symptoms of the disease upon infection including necrosis accompanied by oozing (evident at the posterior side of leaves) in addition to brown (*in planta*) and dark brown to black (detached leaves) de-colorization of the leaves.

Figure 6 (B). Pathogenicity test performed for the validation of pathogen. (d-e) Pathogenicity test on detached leaves and control.

Twig blight is a very complex disease, its whole life cycle occurs in a close association of host plant, where it is able to infect flower tissue, shoot, leaf and fruit. In this study the virulent isolates (Tax2a, Tax11b Wah13c) showed an increased progress of symptoms on inoculated shoots as compared to other inoculated shoots. This whole process of symptom appearance and the variation among the production of symptoms may be accompanied by the production of difference in levels of exopolysaccharides, and other infection concomitant proteins i.*E. amylovora*n, and the T3SS system, which enables the bacteria to enter host tissues and cause infection (Vrancken *et al.*, 2013). The results are also in accordance with Slack *et al.* (2017). He observed similar necrosis after incubating inoculated detached leaves.

PCR Optimization of Pathogenic Isolates

The three most virulent isolates viz: Tax2a, Tax11b Wah13c, confirmed by pathogenicity testing, were chosen for multi-locus sequence analysis. 100% homology was shown by all isolates with already submitted sequences of *Erwinia amylovora*. PCR reaction and conditions were optimized for the successful amplification of *Erwinia amylovora*. After the successful visualization of the DNA samples of the three most virulent isolates through gel electrophoresis, the samples were submitted to Macrogen Korea for sequencing. After sequence submission to gen bank, the accession numbers along with their length of fragments were obtained as shown in Table 3, and the bands obtained after PCR amplification of three virulent isolates obtained after pathogenicity test with two chromosomal and one plasmid gene.

Phylogenetic Analysis of *Erwinia amylovora* Using Chromosomal and Plasmid pAE 29 genes

Evolutionary history was inferred using the Maximum Parsimony method.

Out of 9 most parsimonious trees for both genes (length = 50 & 100 respectively) are shown in figure 7 and 8. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (pg. 126 in ref. (Nei and S., 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). This analysis involved 16 nucleotide sequences. There was a total of 29593 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. A phylogram was generated using the sequences of chromosomal and plasmid genes

(Figure 8). The reference sequences were obtained from GenBank. The isolates (in bold) of *Erwinia amylovora* showed 100% homology with already submitted sequences of GenBank.

Table 3. Molecular characterization of *Erwinia amylovora.*

Primer Name	Sequence	Accession Numbers	Length of Fragments
pstS glmS	CCGAAGAACGATTGCACTAC CGGTTAGTTAGCGCAGTCTC	MN915084	900bp
amsB amsB	GCTACCAGCAGGGTGAG TCATCACGATGGTGTAG	MN902191	1600bp
pAE29F pAE29R	TTCACGGCTTCGCAGAT ACCCGCCAGGATAGTCGCATA	MN902192	713bp

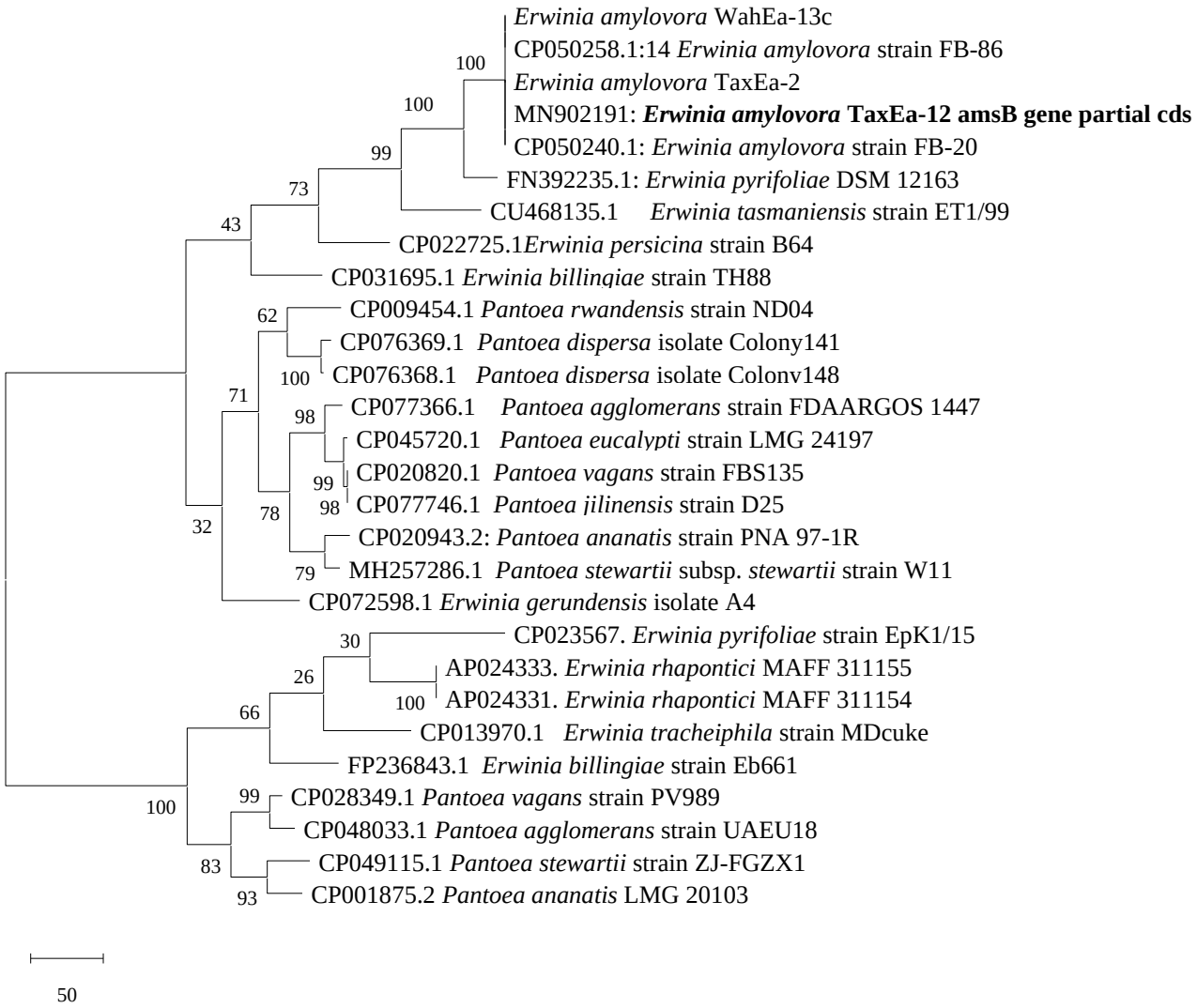

Table 7. Phylogenetic analysis of *Erwinia amylovora* using amsb gene.

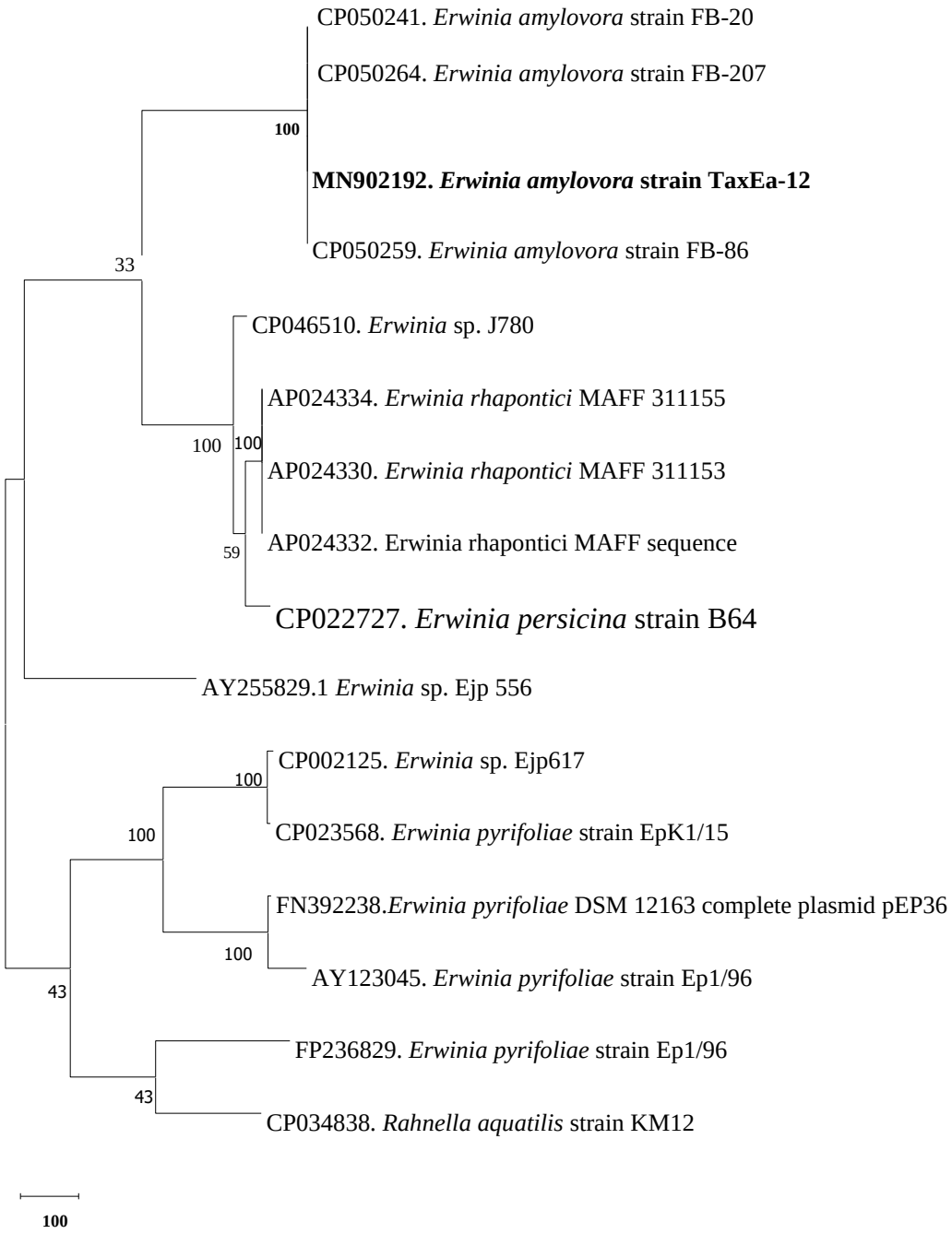

Figure 8. Phylogenetic analysis of *Erwinia amylovora* using Plasmid pAE 29.

Erwinia amylovora is considered as a homogenous species, as far as the host range is concerned they can only vary in their pathogenic capability such as an increase or diminution in an acquired bacterial ability to infect plant species, which were initially resistant to the disease instigated by this particular bacterium (Puławska and Sobiczewski, 2012; Jones and Schnabel, 2000), but this phylogenetic analysis conducted for both the chromosomal genome & plasmid genome of *Erwinia amylovora* strains exhibiting much greater level of genetic diversity in chromosomal region as shown in Figure 6 and 7 than the plasmid genome. This variability

might be due to the variation in environmental conditions, that may lead to the altered phenotypic hereditary characteristics, and a modified genetic material (Agrios, 2005).

AUTHOR CONTRIBUTIONS

Alveen Mumtaz conceived the research idea, performed the experiments, and wrote the manuscript. Farah Naz supervised the research project as the doctoral supervisor. Gulshan Irshad and Riffat Hayat served as members of the supervisory committee.

CONFLICTS OF INTEREST

Authors declare that there are no conflicts of interest.

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