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Bacterial identification causing soft rot and blackleg diseases of Tomato and Onion in district Mansehra, Pakistan

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ABSTRACT

Vegetables are essential for the growth of the human beings. It provides vitamins, antioxidant, fibers and other nutrients essential for the human health. Nearly 70% population of Pakistan depends directly or indirectly on agriculture. Due to the favorable climatic conditions, Mansehra district is included in the list of vegetable production areas. However, certain factors lower the quality and quantity of vegetables in which bacteria play a key role. We collect the samples from the farmer fields and then investigated the causal pathogens. Then further, pure colonies were obtained which were subjected to PCR and *16S RNA* gene was sequenced. We found that causal agents were *Pectobacterium* sp., *Erwinia* sp. and *Pseudomonas* species. The sequences obtained from *16S RNA* were submitted to NCBI Gene Bank having Accession numbers MH265998.1, MH251625.1 and MH263738.1.

Keywords: Onion (*Allium cepa* L.); Tomato; Molecular identification; *16S RNA;* PCR; Sequence analysis

Introduction

Vegetables are the prolonged plants parts that are used in different ways and vital for the health and growth of the human beings (Yusuf, Oyaweye, Yongabi, & Pemu, 2004). These vegetables are regarded essential for healthy life because it provides essential nutrients and others macro, micro nutrients required for growth and functions.

Onion (Allium cepa L.) is the important crop cultivated throughout the world. The Allium belongs to family Amaryllidaceae. It is included in perennial herbs having underground bulb. This genus also include garlic, chives and certain other non-edible ornamental species (Mollavali et al., 2016). The common onion has variety in flavor and color i.e. red, yellow and green. It can be used either cooked or uncooked (Van Wyk, 2014). It is valuable crop having important chemical constituents like water moisture, carbohydrate, total sugar, vitamin C, calcium, phosphorus, potassium, protein and fats (Bhattacharjee et al., 2013). Moreover, the onion has antioxidant activity and reduces the risk of cancer and other cardiovascular diseases. It is also reported that onion have antiasthmatic, hypotensive and antispasmodic properties (Stajner & Varga, 2003). The onion has unpleasant smell which is due to the chemical named allylpropyl disulphid which have medicinal properties regarding digestion, eye and heart stimulation (Baloch, 1994; Shanmugavelu, 1989).

The onion is consumed in greater amounts and regarded as a valuable crop worldwide (Ali, Parikh, & Shah, 1994; FİDAN & KOÇ, 2001). In Pakistan's GDP, the onion plays substantial role. It is cultivated on an area of 128900ha area with a production of 1966920 tons throughout Pakistan in which Khyber Pakhtunkhwa contributes about 193870 tons (PAKISTAN, 2015-2016).

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Tomato is cultivated and consumed worldwide. Tomato like onion are used either cooked or may be used in salad, sauce as uncooked (Lohano & Mari, 2005). Tomato have high nutritional value having vitamin A, E and C which are important for human life. It also contains water, calcium and niacin, all of which have essential role in metabolic activities of human beings (Jaramillo, Rodriguez, Guzman, Zapata, & Rengifo, 2007; Olaniyi, Akanbi, Adejumo, & Ak, 2010).Tomato is one of the rich source of antioxidant, several important minerals and fibers which are indispensable for human health. Various genes are present in different variants of tomato which control different protein responsible for the synthesis of lycopene and cyanine which are antioxidant in property (Stahl et al., 2001).

Tomato are cultivated on an area of about 63200ha with a production of 599700 tons throughout Pakistan in which 135700 tons are contributed by Khyber Pakhtunkhwa province which are cultivated on an area of 1400ha (PAKISTAN, 2015-2016).

Different pathogens including bacteria, fungi, virus attacks on vegetables. it is reported that about one hundred bacterial species have pathogenicity to cause diseases in plants (Bull et al., 2014; Jackson, 2009; Martins, Merfa, Takita, & De Souza, 2018; Sundström et al., 2014). *Erwinia (Pectobacterium)* is the included in the list of the pathogens that causes great loss to the vegetables (Mansfield et al., 2012; Tesoriero, 2018; Zaczek-Moczydłowska, Fleming, Young, Campbell, & O'Hanlon, 2019). The *Erwinia* genus is included in Enterobacteriaceae family which have several species known up to now which cause rotting of vegetables and certain other economic crops (Adeolu, Alnajar, Naushad, & Gupta, 2016). The genus *Erwinia* includes *carotovora* subsp. *atroseptica*, *carotovorum* subsp. *brasiliensis*, *chrysanthami* and *Erwinia wasabiae*. In all of these, the *carotovora* and *atroseptica* species are the prominent for the loss of vegetables (Nykyri et al., 2012; Pitman, Harrow, & Visnovsky, 2010). While the specie *chrysanthami* are reported to have tolerance for both warm and cold environmental conditions (Hannukkala & Segerstedt, 2004).

Bacteria are cosmopolitan in nature and have wide host range. Erwinia carotovora subsp. brasiliensis was reported from Brazil to cause soft rot and blackleg of potato (Duarte, De Boer, Ward, & Oliveira, 2004; Kannan, Bastas, & Devi, 2015). It is documented that Erwinia carotovora subsp. atroseptica (Ecc) can cause pathogenicity at temperate region while Subsp. carotovorum can cause damage at both higher and lower temperature (Ozturk, Aksoy, Potrykus, & Lojkowska, 2018; Wells & Moline, 1991).

Onion and tomato are important cash crops cultivated in Mansehra, Pakistan. It is highly prone to bacterial pathogens. For this purpose, disease free seed is important because it is mainly seed born. The use of PCR and sequencing are precise in the identification. The gene sequencing is efficient in the identification of bacterial pathogens so that we can get disease free seed and in turn the disease free crops.

Literature review

Bacteria is one of the devasting pathogens and cause severe damage to the vegetable crops by reducing the quality and quantity of the vegetable. Due to the pathogenicity a lot of work has been

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done on bacteria which include ecology, severity and distribution of the pathogens by variety of ways. They are reviewed as under;

Study area

The study area was selected Mansehra, Pakistan because here mostly vegetables are cultivated rather than cereal crops. The Mansehra is located in 34°-15 and 35°-12 at North latitude. It is adjacent to Abbottabad, Bunnir and Battagram districts (Figure.1) (SMEDA, 2009). Mansehra is regarded as economic district of Khyber Pakhtunkhwa due vegetable production and here two growing seasons occurs. Kharif which starts from April to September and Rabi which starts from October to March. Due to its fertile soil and favorable conditions onion and tomato yields are high in the district Mansehra (Shah & Khan, 2012).

Major diseases

Pakistan is an agricultural country and mainly depends on agriculture which reduces unemployment in greater ratio. It is estimated that about 60% of Pakistan population earn through the agriculture and farming. However, there are many problems related to agriculture in which prominent is pathogen attack which disturb the quality and quantity of the crops to about half as compared to that of developed countries causing loss of above $1bn dollars per annum to the food production (Chaudhry, Kayani, & Salam, 1991; Kannan et al., 2015; Qureshi, 2002).it is reported that nearly hundred bacterial species can cause pathogenicity in plants (Bull et al., 2014; Jackson, 2009). These bacteria causes blackleg, soft rot, ring rot and bacterial scab which reduces the market value of the vegetables (Charkowski, 2018). It is reported that *Ralstonia solanacearum* is well known cause of wilt and brown rot in Tomato in Khyber Pakhtunkhwa province causing 30% yield loss with about 30% *Erwinia carotovora* subsp. *atroseptica* and *Erwinia carotovora* subsp. *carotovorum*. The Erwinia pathogen is a great challenge which are included in top ten of bacterial pathogens which cause great loss to crops (Mansfield et al., 2012; Nion & Toyota, 2015).

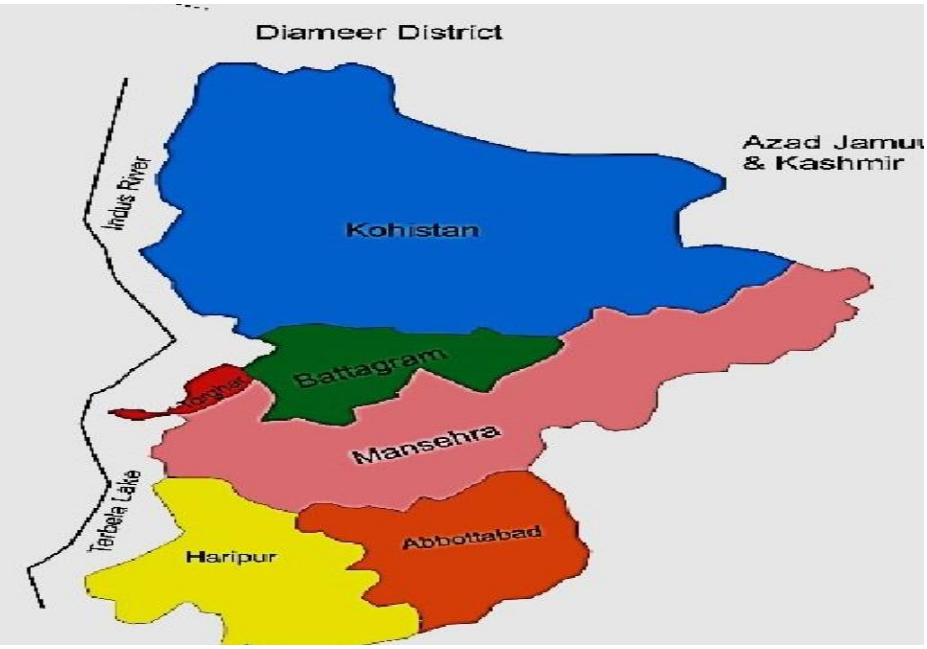

Figure 1. Map of Mansehra showing boundaries with other adjacent districts of Khyber Pakhtunkhwa, Pakistan.

Soft rot and Black lag

Soft rot of vegetables is due to bacteria *Erwinia carotovora* subsp. *carotovorum* (*Ecc*), *E. carotovora* subsp. *atroseptica (Eca*) and *E. carotovora* subsp. *chrysanthami* (*Ech*) (Perombelon & Kelman, 1980). These pathogens secrete pectinase that digest middle lamella resulting in soft rot (Barras, van Gijsegem, & Chatterjee, 1994; Collmer, Berman, & Mount, 1982; Collmer & Keen, 1986; Salmond, 1994). It is reported that *Erwinia* as *carotovora* subsp. *atroseptica, carotovora* subsp. *carotovora* and *chrysanthami* are different genetically and have different pathogenicity which result blackleg, aerial stem rot and soft rot (Fikowicz-Krosko & Czajkowski, 2017). For the pathogen establishment the interaction among the bacterial strain and the host plant is established in which environmental conditions plays important role in disease development. Optimum temperature and high rate of humidity favors the disease establishment.

It is reported that mostly the pathogenic bacteria move with the help of flagella and the gene which are responsible for this movement code for proteins that responsible for virulence to pathogen-host interactions (Van Vaerenbergh, Baeyen, De Vos, & Maes, 2012). Different techniques which infer the relationship among the pathogens of family Enterobacteriaceae, which includes morphological, biological and molecular methods (Czajkowski et al., 2015). Molecular study is more precise in which PCR plays a key role in the identification and bacterial classification. It was reported that cellulose and other pectic substances reduce the reliability of the PCR (Arulappan et al., 1996; Elphinstone, 1996).

Material and Methods

Collection of bacterial samples

In order to record the occurrence, severity and distribution of the pathogens various field survey were made in Mansehra district in summer, winter and autumn. Two to three points of size 15-20ft long in each survey. Then calculation was made as total number of plants per point and infected plants accordingly. This was analyzed for the control of pathogen and for management study of cultivation of plant in respective fields. Moreover, the infected plants parts (Figure 2) were collected from fields, labelled and transported to Molecular Genetics laboratory, Hazara University, Mansehra.

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Figure 2. Symptomatic samples at the farmer's fields. (A) Tomato (B) Onion

Isolation of the pathogen

The leaves, fruits and bulbs of the tomato and onion were cuts into slices and then with distilled water surface sterilized. Then grinded in 0.84% sodium chloride solution due to which suspension was formed which is then transferred to test tube having was 1ml distilled water. Then from test, bacterial streaking was done on nutrient agar. The plates were inverted and placed at temperature of 28-30 °C for 36 to 48 hr. After this, different colonies arose on nutrient agar in which there was also whitish creamy color and non-flat. These colonies of creamy color were selected and transferred to another fresh medium petri plates. After several repeat pure colonies were obtained (Figure 3). Nutrient agar is non-specific and every type of microorganism tends to grow including fungus. For fungus free culture Fungone having Fluconazole were used. The pure colonies after the culture were selected for further studies by toothpick and stock in 75% glycerol on -20 °C. For re-culturing the bacteria from the stock were streaked on nutrient agar and kept 28-30 °C for 2days.

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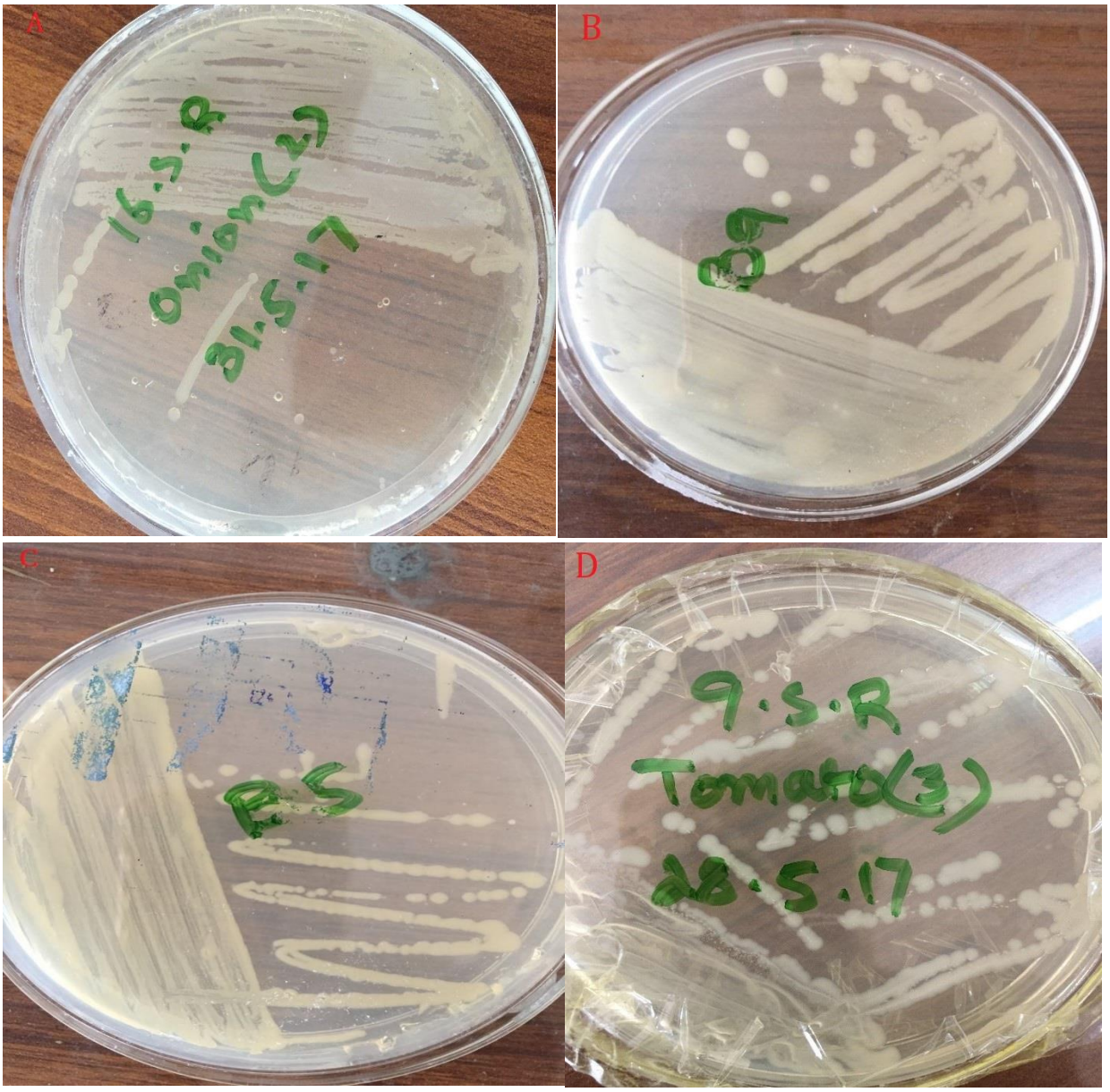

C D

Figure 3. The colonies of isolated bacteria. (A) Unpurified bacteria isolated from onion (B) Purified colonies isolated from onion (C) shows unpurified colonies isolated from tomato while (D) shows purified colonies isolated from tomato.

DNA Extraction

For DNA extraction, the bacterial isolates at 28°C in 5ml broth medium for overnight were cultured in chamber. Then 1.5ml were taken in Eppendorf tube centrifuge at 8000rpm for 10min, removing supernatant and then dissolved in 100µl ddH_2O. According to (De Boer & Ward, 1995), the bacterial cell are break down by heat which is called water boiling method. It is easy and precise technique to isolate DNA from bacteria. Little change in previously filed method was made and the water was boiled for 95°C for 5-6min having single colony of bacteria. Then directly transferred to -20°C which is ready for further research work and used 5 µl as a template in PCR.

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Molecular study

By morphological characters we can classify the bacterial pathogens but sometime similarities in the species occurs which are difficult to differentiate. So by genome level study we can classify the bacterial pathogens which are more precise and accurate than the ordinary morphological techniques. Due to this we used PCR of applied biosystems 2720 of life technologies. For PCR we used universal primers as Forward 9F (GAGTTTGATCCTGGCTCAG) and Reverse 1510R (GGCTACCTTGTTACGA). also we used the specific primers for the *Erwinia carotovora* subsp. *atroseptica* Eca1F (CGGCATCATAAAAACACG) and Eca2R (GCACACTTCATCCAGCGA) as described by (De Boer & Ward, 1995). The enzymatics kit was used and 10µl reaction was used with the ratio (Table 1) and (Figure 4).

Table 1. List of Master Mix and their quantity used in PCR during this study.

S.NO.	Ingredients	Each reaction (1x)
1.	10x Buffer (Mg^+ free)	1 µl
2.	dNTP	1 µl
3.	$MgCl_2$	0.75µl
4.	Taq polymerase	0.2 µl
5.	Primer-F	0.5 µl
6.	Primer-R	0.5 µl
7.	Template	5 µl
8.	ddH_2O	1.05 µl

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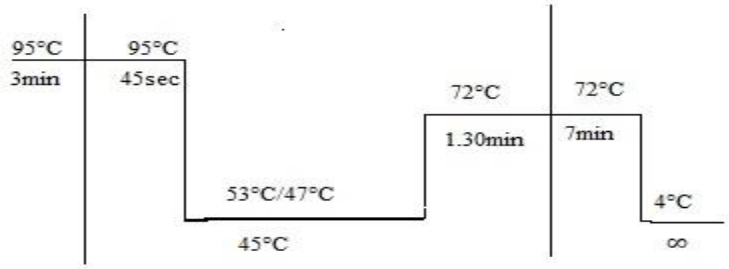

Figure 4. PCR profile for the gene specific and *16S RNA* primers.

Gel Electrophoresis

The agarose gel of 1.5% was made to visualize the PCR product. The agarose was dissolved in TAE buffer and placed in oven for about 3min and placed at room temperature for about 40min. then poured the liquid agarose into the medium size tray having comb for the well fitted. Then after mixing with loading, 5µl PCR product was load and run at 80volts for 50min along with ladder. Then Gel Doc of Biodigonstic was used to visualize the product. In this process UV and ETBr (Ethelium bromide) is carcinogenic needs care.

Purification of gene

After the 16S RNA universal primers at the applied biosystems 2720 Thermal cycler of life technologies the product was run at 1% agarose gel and then the band was cut and visualize under UV light for gene cleaning. Then transferred the band into 1.5ml Eppendorf tube. Sanprep column DNA gel extraction kit (Sangon Biotech) was used for purification.

Sequencing of purified gene

The purified product was then sent to MACROGEN, Korea for sequence analysis. The result given by MACROGEN, Korea were analysed with different ways for further investigation and identification.

Results

For the pathogenicity of pathogens different fields were visited at different seasons of the year. The tomato and onion crops were greatly infected in some areas which were difficult to purify its pure culture. It mostly depends on the temperature of the area which favors the growth of bacteria. So, *Erwinia carotovora* subsp. *atroseptica* were found at high altitude where temperature is lower causing blackleg and soft rot. The *E. carotovora* subsp. *carotovorum* were found destructive at

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higher altitude which favors higher temperature. Moreover, we also found *Streptomyces* sp., *Bacillus* sp, *Pseudomonas* sp. and *Enterobacter* sp. in our research.

Molecular results

The use of specific primers to Erwinia carotovora subsp. atroseptica was used as Eca1F and Eca2R give 690bp bands. This was used because some characters are similar due to which differentiation becomes difficult. In this method the Taq polymerase plays a key role. The PCR bands are shown in Figure 5.

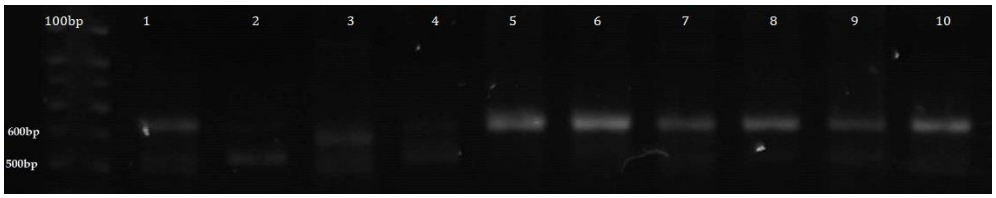

Figure 5. amplification pattern of Eca specific primers by using PCR. The specific primers for Eca produce 690bp band. At the left of agarose (1.5%) gel 100bp DNA ladder was used.

Sequencing

After purification of 16S RNA bands the product of 15 µl was sent to MACROGEN, Korea for sequencing. The given result of MACROGEN, Korea were submitted to NCBI Gene Bank with accession No MH265998.1, MH251625.1 and MH263738.1 which shows similarity with *Pectobacterium* sp., *Erwinia* sp. and *Pseudomonas* sp. respectively which are regarded destructive for the Vegetables cultivated in Mansehra district.

>**MH265998.1**

GGGGCATGGACGGGGGGTCTACACATGCAGTCGAGCGGCAGCACAGAGAGCTTGCT
CTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGG
GGGATAAGTACTGTAATCAATGGTTAAGACCGCCTGCTCGCCTACCATCATTCTGTT
GAACCCCAGATCCTCATGGCCTCAAAGGTCCGCAACCGGGATCACGTGGCTGCTTTC
CGGCCACTGCCGCTCATCGGCGATCCCTAGGTCATACGGCATTTCCCCCGCCCGCTG
TGCAATTGAATGCTCAGGCATAATGCGACTCTGGCTGTCGATGACGACGATTAGCGG
CTGGCGCCAATTCTCTTGGGGATAAACCGCCTGGGTATCGGCGGTCAGCTCTTGCCA
TCTCACGGTCAACGCCCGATCGTTCCCAACCTCGGCGGCGCTGCTGTTTATGATGTC
TGGTTTTGTCTCGTTGACTCTTCACTTCCCGTTTCCCGTGCACCTATGTGATCTCTGCG

Dendrogram of MH265998.1

The Dendrogram of MH265998.1 (Figure 6) representing the comparison of DNA sequence amplified through PCR by using *16S RNA* primers. According to the tree the sample is closely related with *Pectobacterium* species.

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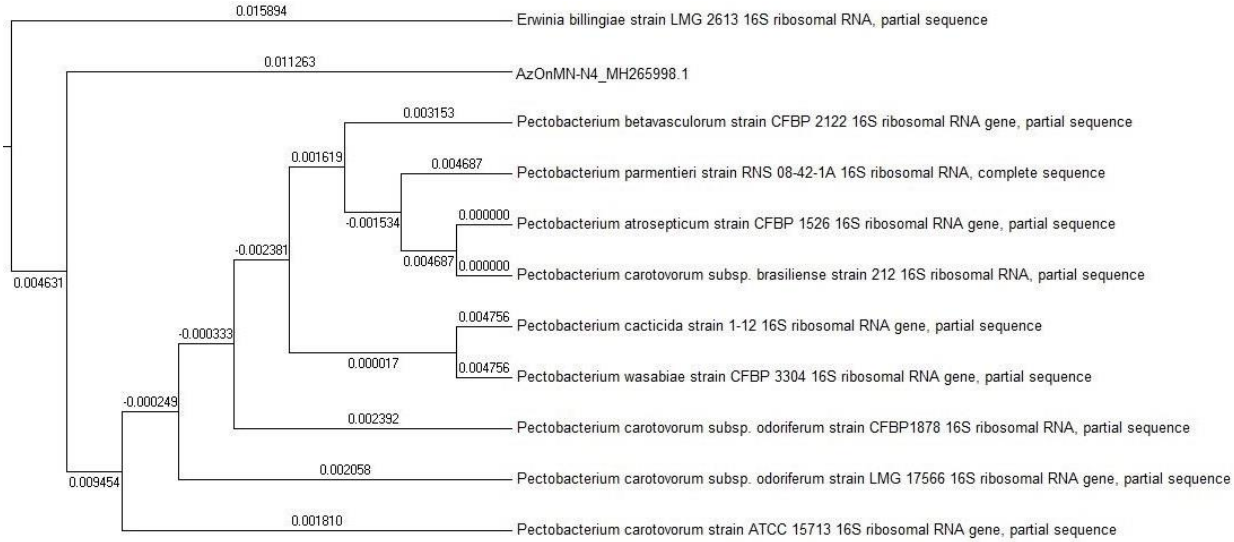

Figure 6. Phylogenetic tree of sample MH265998.1

> **MH251625.1**

AGGGCGGCAGGCTACACATGCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGT
GACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAA
CTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAGACCAAAGTGGGGGACCT
TCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAAC
GGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGA
ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG

Dendrogram of sample MH251625.1

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The Dendrogram of MH251625.1 (Figure 7) representing the comparison of DNA sequence amplified through PCR by using *16S RNA* primers. According to the tree the sample is closely related with *Erwinia* species.

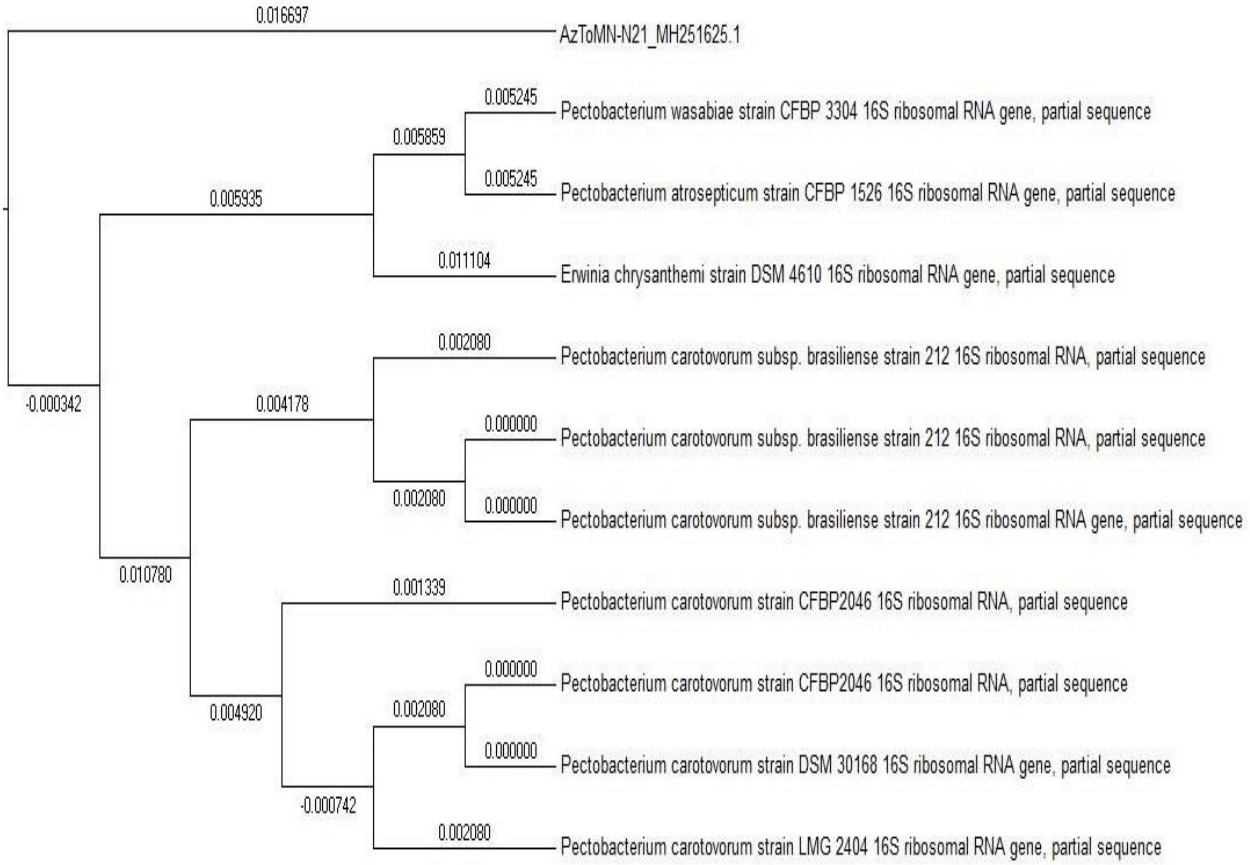

Figure 7. Phylogenetic tree of sample MH251625.1

> **MH263738.1**

AGGGCGGCAGGCTACACATGCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGT
GACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAA
CTACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAGACCAAAGTGGGGGACCT
TCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAAC
GGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGA
ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG

Dendrogram of sample MH263738.1

The Dendrogram of MH263738.1 (Figure 8) representing the comparison of DNA sequence amplified through PCR by using *16S RNA* primers. According to the tree the sample is closely related with *Pseudomonas* species.

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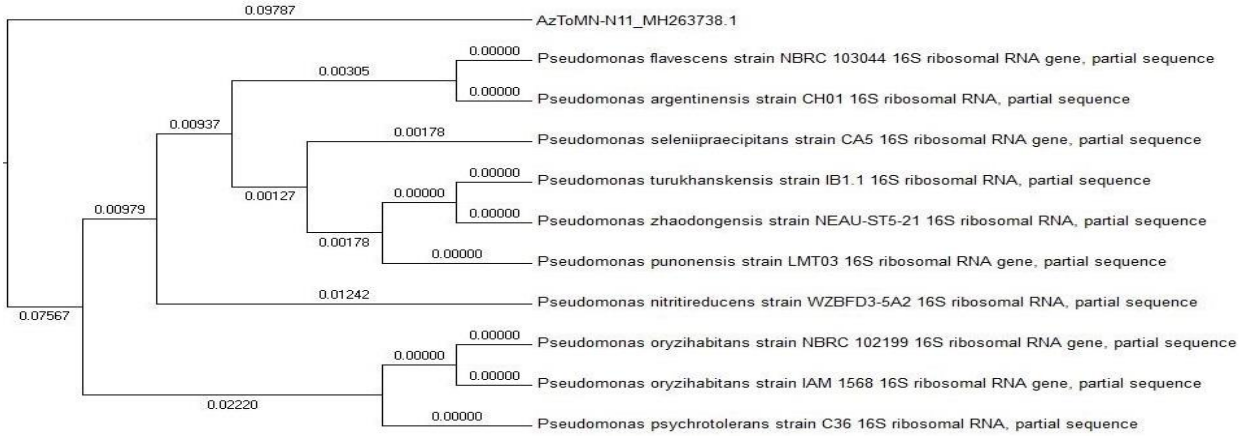

Figure 8. Phylogenetic tree of sample MH263738.1

Discussion

We visited different farmer fields on which tomato and onion were cultivated. In these fields sample from the infected vegetables were collected. It was observed in our field survey that at hilly area where the altitude was high the blackleg and soft rot was in abundance which is due to *Erwinia carotovora* subsp. *atroseptica* as described by (Pérombelon, 1979). At lower altitude where temperature is slightly higher than other elevated area the pathogenicity was due to *Erwinia carotovora* subsp. *carotovorum* (Ecc) as filed by (Oliveira, Duarte, Silveira, & Moraes, 2003). We also found the *Streptomyces* sp., *Bacillus* sp. *Enterobacter* sp. These species are found to favor humid and low temperature environmental conditions as described by (Iftikhar, Ahmad, Soomro, & Aslam, 1993).

Conclusion

Vegetables are essential for the life of human beings. They provide enough supply of vitamins and others essential nutrients required for human health. The climate of Mansehra is suitable for the vegetables. so, the current work was to find the challenges to the cultivation of vegetables in Mansehra district. Here the vegetables are cultivated in sufficient amount the profit gain is less due to bacterial rotting. Proper pathogens free seed to be provided to the farmers so that they can grow disease free crops. The crop rotation will play efficient role because mainly these pathogens are soil born.

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