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Effect of temperature and inoculums level on development of soft rot of potato caused by *Erwinia carotovora* subsp. *carotovora* and their molecular detection through poymearse chain reaction

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Abstract
Erwinia carotovora ssp. *carotovora* (Jones) (Bergey *et al.*, 1939) (now known as *Pectobacterium carotovorum* subsp. *carotovorum*) are the causal agents of soft rot of potato. These diseases represent a serious threat to potato production and post harvest losses during storage in warm and temperate climates. The study on effect of different inoculums levels and temperature reveal that maximum rotting *i.e.* 83.33 % was recorded at 35 ºC followed by 45% at 28 ºC and 26.67% at 21 ºC temperature at 10^8 cfu/ml inoculums level. The sensitive and early detection of soft rot pathogen, *E. carotovora* ssp. *carotovora* is key for the avoiding the occurrence of diseases in the field and rotting of tubers in storage. In present study, PCR protocol was developed for the detection of *E. carotovora* ssp. *carotovora* from potato tubers. A set of Oligos targeting the pectate lyase gene of *E. carotovora* ssp. *carotovora* amplified the 418 bp fragment. The sets were able to detect the *E. carotovora* ssp. *carotovora* upto 1.0 ng of genomic DNA and assay on CFU detection, allowed detection up to 10^3 cfu/ml of *E. carotovora* ssp. *carotovora* by PCR. So, detection by PCR methods offers sensitive, specific, reliable and fast detection of soft rot bacterial pathogens from potato tubers. Thus offers a gain in time and materials thereby emphasizes its importance in large-scale detection processes such as pathogen-free seed certification.

Keywords: Soft rot, potato, *Erwinia carotovora* ssp. *carotovora*, PCR, detection, incoulum, temperature etc

Introduction
Potato (*Solanum tuberosum* L.) is the third most important food crop of the world after wheat and rice, which is roughly half the world's annual output of all root and tuber crops. Its production is 330 million tons fresh tubers from 19.7 million hectares with a productivity of 17.0 t/ha (FAOSTAT, www.faostat.fao.com). Potato yield per hectare differs largely from country to country due to the climate, national agricultural policy, differences in the manner of potato cultivation and also because of the presence of potato diseases affecting plant growth and tubers during storage (Czajkowski *et al.*, 2012) [6].

Pectinolytic bacteria, *Erwinia carotovora* ssp. *carotovora* (Jones) (Bergey *et al.*, 1939) [3] (now known as *Pectobacterium carotovorum* subsp. *carotovorum*) are the causal agents of soft rot of potato. These diseases represent a serious threat to potato production and post harvest losses during storage in warm and temperate climates. Soft rot of vegetables caused by various species of *Bacillus*, *Pseudomonas* and *Erwinia* (Agrios, 2006) [1]. *Erwinia carotovora* subsp. *carotovora* is however considered to one of the major soft rot causing bacterium (Larka, 2004) [14]. Soft rot (*Erwinia carotovora* subsp. *carotovora* (Jones) (Bergey *et al*., 1939) [3] is of great importance both in field as well as in transit and storage, causing heavy economic losses to various vegetables. Post harvest bacterial soft rot losses have been estimated to vary between 15-30% of the harvested crop (Agrios, 2006) [1]. These bacteria remain latent for a long time in asymptomatic potato tubers, which are one of the main factors for the disease's dissemination (Ciampi *et al*. 1981; Zielke and Naumann 1984) [5, 30]. It has the broadest host range and the ability to survive in different environments both inside and on a wide range of alternate hosts (Perombelon and Kelman, 1980; Sledz *et al*., 2000) [21, 26]. Repeated attempts to breed for resistance to these bacteria in potato using wild *Solanum* spp. have not been successful yet (Birch *et al*., 2012; Lebecka *et al*., 2006) [4, 5]. Moreover, the disease control under field conditions based on physical, chemical and biological methods has also failed (Czajkowski *et al*., 2012) [6].

As seed tubers are among the main source and vector of inoculums of soft rot bacterial pathogens (Hélias *et al*. 2000) [11], and since no chemical control of the pathogen exists, disease prevention methods are limited to sanitary measures and the use of certified pathogen-free seed tubers (Janse and Wenneker, 2002) [13]. Certification necessitates large-scale and extensive detection techniques. Molecular detection methods including immunological and PCR based techniques are the most rapid and accurate approaches (Ward *et al*., 2004; Alvarez, 2004; Singh *et al*., 2014) [29, 2]. PCR-based techniques allow selective, rapid and sensitive and accurate detection of *Pectobacterium* spp. (Hyman *et al*., 2001) [12]. In spite of much research, no effective chemical treatment (Diallo *et al*., 2009; Evans *et al*., 2010) [8, 9] nor resistant cultivars are available for controlling potato soft rot (Rasche *et al*., 2006) [24]. Potato shippers (producers and traders) need information that would enable them to detect the soft rot causing pathogen *E. carotovora* subsp. *carotovora* from asymptomatic potato tuber/plant samples.

Therefore, the current practical approach is based on phytosanitary measures for the production and multiplication of pathogen-free potato seed stocks (Czajkowski *et al*., 2012) [6]. This involves seed certification programmes to verify seed health on during field inspections and laboratory tests using reliable and sensitive molecular techniques when assessing seed tuber contamination incidence (Toth *et al*., 2011) [27, 28]. The purpose of this study was to develop a specific and sensitive polymerase chain reaction (PCR) assay for the rapid, accurate and detection of *E. carotovora* subsp. *carotovora* in asymptomatic potato tuber/plant samples, which will be helpful to save time and money.

Materials and Methods

Culture collection and maintenance on media: Pectinolytic bacteria *E. carotovora* subsp. *carotovora* causing soft rot disease in potato were found to be associated to cause spoilage in tubers during storage. Soft rot causing bacteria *E. carotovora* subsp. *carotovora*, strain NAIMCC – B00296 was obtained from National Bureau of Agriculturally Important Microorganism (ICAR), Kushmaur, Mau (U.P.). The culture was grown and maintained on nutrient agar medium.

Pathogenicity test of *E. carotovora* subsp. *carotovora*: The healthy potato samples free from bruises, rot, wound etc were washed thoroughly in running tap water followed by distilled water. The tubers were surface sterilized with 10% sodium hypochlorite solution, followed by washing with sterile distilled water. Tubers were allowed to dry and wounded by punching one hole about 10 mm deep using 5 mm thick cork borer. The 48 h old culture of *E. carotovora* subsp. *carotovora* containing 0.1 O.D inoculated to potato and kept in glass jar. Relative humidity was maintained inside the jar by putting sterilized cotton swab. The inoculated potato samples were kept for incubation at 35 ºC and observation of rotting was recorded after 48 h of inoculation.

Artificial inoculation of *E. carotovora* subsp. *carotovora* on potato tubers through different population level and temperature: To confirm the role of *E. carotovora* subsp. *carotovora* on rotting of potato, an experiment was conducted by artificial inoculation of *E. carotovora* subsp. *carotovora* through different population level. Healthy, uniform, homogenous potatoes tubers were collected from the market and brought to the laboratory. Potato tubers were washed thoroughly in running tap water followed by distilled water. Prior to inoculation, the tubers were surface sterilized with 10% sodium hypochlorite solution for three min, followed by washing with sterile distilled water. Tubers were allowed to dry at room temperature. After drying, the tubers were wounded by punching one hole about 10.0 mm deep using 5.0 mm diameter thick cork borer. Simultaneously, the inoculums suspensions of *E. carotovora* subsp. *carotovora* were prepared in sterilized distilled water from 48 h old culture. The concentration of bacterial suspension was 0.1 OD at 600 nm in spectrophotometer considered as 10^8 cfu / ml inoculums population. Inoculums load population was prepared10^2, 10^4, 10^6 and 10^8 by serial dilution of *E. carotovora* subsp. *carotovora*. 200 µl of *E. carotovora* subsp. *carotovora* was inoculated into the hole made on the potato tubers separately from each population level *i.e*. 10^2, 10^4, 10^6 and 10^8 cfu/ml. After inoculation, the inoculated hole of tubers was covered with sterilized wet cotton to maintain the relative humidity. Further, the all tubers were kept in closed glass jar, in which humidity was maintained by using same sterilized wet cotton in bottom of the jar and on the platform of glass jar inoculated tubers were kept. Finally, the inoculated set of glass jars were kept at 21, 28 and 35 ºC temperature with three replications. Observation of potato rotting was recorded after 3 days of inoculation. Rotting of potato was rated by estimating the percent rotted area of tuber cut lengthwise. This criterion was more consistently applicable than the diameter of rotted area (DeBoer and Kelman, 1978) [7], since the decayed area was not always circular or semicircular. An average value from three tubers per replication was used for statistical analysis.

DNA extraction: The culture of *E. carotovora* subsp. *carotovora* was inoculated in nutrient broth and grown at 28±2 °C for 48 h with shaking at 200 rpm. 48 h grown culture of *E. carotovora* subsp. *carotovora* were taken in 2 ml eppendorf tubes and the total DNA of *E. carotovora* subsp. *carotovora* extraction done by CTAB method (Murray and Thompson, 1980) [20].

Designing of primer for *E. carotovora* subsp. *carotovora*: A set of primer EF 5'- GCAGCAGGAAGCCTACAAAC-3' & ER5'-ACAGCCATCCTCAAAATCGT-3' was designed from locus of pectatelyase gene (*pel)* having product size 418 bp of *E. carotovora* subsp. *carotovora*, using Primer 3 program (www.frodo.wi.nit. edu) and specificity was checked by in silico using website (www.insilico.ehu.es). The developed primers were validated for their universality across *E. carotovora* subsp. *carotovora* and other related group of bacteria by primer blasting in NCBI website (www.ncbi.nlm.nih.gov).

Standardization of protocol for detection of *E. carotovora* subsp. *carotovora*: Protocol for detection of *E. carotovora* subsp. *carotovora* was standardized. The optimized primer concentration and PCR conditions of designed primer to detect the *E. carotovora* subsp. *carotovora* was performed in a volume of 25 µl containing, 5.0 µl of 5X Taq buffer, 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs,0.5 µl each 10 pmoles of the primers EF and ER, 1 unit Taq polymerase (Promega) and 1 µl of 100 ng DNA templates was used. Amplification was performed in a BIO-RAD C1000 thermo cycler. The PCR products were resolved using a 1.2% agarose gels stained with ethidium bromide at 0.5 µg/ml, and photographed under UV lighting at gel documentation system.

Sensitivity and specificity of the primer of *E. carotovora* subsp. *carotovora*: To determine the sensitivity of the primer 100 ng genomic DNA of *E. carotovora* subsp. *carotovora* was serially diluted up to 0.001 ng was serially diluted in nuclease free molecular water. The prepared serial dilutions of the purified genomic DNA were directly used for the PCR assay as per the protocol described for *E. carotovora* subsp. *carotovora* above.

To test the specificity of the newly designed primers EF/ER for detection of *E. carotovora* subsp. *carotovora*, different groups of bacteria such as *R. solanacearum, Pantoea agglomerans, Pseudomonas fluorescens, Xanthomonas oryzae* pv. *oryzae*, *X. campestris* pv. *punicae*, *X. campestris* pv. *campestris* and *Bacillus subtilis* were tested along with *E. carotovora* subsp. *carotovora*. The PCR protocol follows as described for *E. carotovora* subsp. *carotovora* above.

Result

Collection, maintenance on media and pathogenicity test of *E. carotovora* subsp. *carotovora*: The *E. carotovora* subsp. *carotovora* strain NAIMCC – B00296, causing soft rot disease in potato was obtained from National Bureau of Agriculturally Important Microorganism (ICAR), Kushmaur, Mau (U.P.). The culture was grown and maintained on nutrient agar medium and further confirmed by pathogenicity test on potato. The pathogenicity test of *E. carotovora* subsp. *carotovora* causes the soft rot in inoculated potato after 48 h of inoculation. It was also confirmed by colony PCR, using *E. carotovora* subsp. *carotovora* specific designed primer EF/ER amplified at 418 bp.

Artificial inoculation of *Erwinia carotovora* subsp. *carotovora* at different population level and temperature: To determine the population level of *Erwinia carotovora* subsp. *carotovora* in potato rotting at different storage temperature, an experiment was conducted by artificial inoculation of *E. carotovora* subsp. *carotovora* through different population level and incubated at 21, 28 and 35 ºC temperatures. Maximum rotting of potato tuber was found in *E. carotovora* subsp. *carotovora* having 10^8 cfu / ml at 35 ºC (83.33%). *E. carotovora* subsp. *carotovora* was able to cause rotting at population level of 10^2 cfu/ml at 21ºC (Table 1 and Fig. 1). The rotting caused by *Erwinia carotovora* subsp. *carotovora* pathogen was increased significantly by the increasing the temperature and population levels.

Table 1: Rot of potato tuber by artificial inoculation of *Erwinia carotovora* subsp. *carotovora* at different population levels and storage temperature.

Temperature (°C).	Treatment	Potato tuber rot (%)			
		10^2 cfu/ml	10^4 cfu/ml	10^6 cfu/ml	10^8 cfu/ml
21	*E. carotovora* subsp. *carotovora*	11.00	15.00	20.00	26.67
28	*E. carotovora* subsp. *carotovora*	15.00	25.00	35.00	45.00
35	*E. carotovora* subsp. *carotovora*	35.00	58.33	63.33	83.33
	Mock inoculation	0	0	0	0

Statistical Analysis

Treatment	CD Value (5%)	SE (d)
A (Temperature)	1.14	0.57
B (Inoculums level of pathogen)	1.32	0.60
AXB	2.29	1.15

A significant interaction was recorded between incubation temperature and population level of pathogen.

Primer design and standardization of protocol: *E. carotovora* subsp. *carotovora* were confirmed by using specific designed primer EF/ ER (EF 5'-GCAGCAGGAAGCCTACAAAC-3'& ER 5'-ACAGCCATCCTCAAAATCGT-3') which was amplified at 418 bp, following the standardized protocol to detect the *E. carotovora* subsp. *carotovora* was performed in a volume of 25 µl containing, 5.0 µl of 5X Taq buffer, 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs,0.5 µl each 10 pmoles of the primers EF and ER, 1 unit Taq polymerase (Promega) and 1 µl of 100 ng DNA templates was used. Amplification was performed in a BIO-RAD C1000 thermo cycler, with an initial denaturation step at 94 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 45 s; and a final extension step at 72 °C for 5 min.

Specificity of the primer: Designed primer EF 5'-GCAGCAGGAAGCCTACAAAC-3'& ER 5'-ACAGCCATCCTCAAAATCGT-3', to check the specificity of the primers by using in silico-PCR, 418bp nucleotide sequences *E. carotovora* subsp. *carotovora* were obtained. The nucleotide sequences of pectatelyase gene (*pel*) of *E. carotovora* subsp. *carotovora* from NCBI database were matched only with the strains of *E. carotovora* subsp. *carotovora*. The *pel* gene based primer gave a single product at 418bp having DNA as a template of *E. carotovora* subsp. *carotovora*. To test the specificity of the primers, different groups of bacteria such as *R. solanacearum, Pantoea agglomerans, Pseudomonas fluorescens, Xanthomonas oryzae* pv. *oryzae*, *X. axonopodis* pv. *punicae*, *X. campestris* pv. *campestris* and *Bacillus subtilis* were tested along with *E. carotovora* subsp. *carotovora*. Amplification was obtained in *E. carotovora* subsp. *carotovora* at 418 bp. However, none of the other bacteria used in this study amplified with this primer (Fig.2). The primer was found specific for detection of *E. carotovora* subsp. *carotovora.*

Sensitivity of the primer of *E. carotovora* subsp. *carotovora*: The sensitivity of the primers was first evaluated on purified genomic DNA of the pathogen *E. carotovora* subsp. *carotovora*. The serially diluted 100 ng/µl DNA of *E. carotovora* subsp. *carotovora* up to 0.001 ng/ µl was amplified as the protocol described above. PCR results showed that the level of detection of *E. carotovora* subsp. *carotovora* DNA was 1.0 ng/ µl (Fig. 3). Further, the CFU assay on sensitivity of *E. carotovora* subsp. *carotovora* in PCR reaction was evaluated, the bacterial suspension of *E. carotovora* subsp. *carotovora* was able to detect up to 10^3cfu/ml (Fig. 4).

Discussion

Erwinia carotovora subsp. *carotovora* causing soft rot disease in potato were found to be associated to cause spoilage in

tubers during storage. To confirm the role of *E. carotovora* subsp. *carotovora* in potato rotting at different storage temperature and population level, an artificial inoculation of *E. carotovora* subsp. *carotovora* through different population level was done and incubated at 21, 28 and 35 ºC temperatures for 3 days. The maximum rotting of potato tuber was found in *E. carotovora* subsp. *carotovora* having 10^8 cfu / ml at 35 ºC (83.33%) after 3 days of inoculations.

E. carotovora subsp. *carotovora* caused more rotting at 10^8 cfu / ml population levels and cause minimum rotting at population level of 10^2 cfu/ml at 21 ºC. The rotting caused by the pathogen was increased significantly by the increasing the temperature and population level of pathogens. Therefore, the study suggests that *E. carotovora* subsp. *carotovora* mainly involved in rotting of potato during storage even at low population level *i.e.* 10^2 cfu/ml and temperature at 21 ºC.

Raju *et al.* (2008) [23] confirmed the enhancement of rotting ability of the pathogen *E. carotovora* subsp. *carotovora* by increasing temperature of 20 to 30 ºC. They further confirmed that rotting of radish was increased by increasing relative humidity levels and maximum rotting was found at 35 ºC with 100 % relative humidity in raddish discs.

Moh *et al*., 2012 [19] study the influence of temperature (10, 15 and 20 °C), relative humidity (86, 96 and 100%) and initial concentration of bacterial inoculum (10^5, 10^7 et 10^9 CFU/ ml) on the population density of *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* subsp. *carotovorum*. Both bacterial species, statistical analysis showed a significant effect of temperature, relative humidity and initially applied bacterial concentration on population dynamics and soft rot development at the surface of wounded potato tubers. Multiple regression analyses and the contour plots showed that the temperature is the most important factor, followed by the initially applied bacteria concentration and relative humidity.

Potato suffers from many tuber borne fungal and bacterial pathogens and main source of inoculums and dissemination of the diseases from one place to another is through tubers. Soft rot (*E. carotovora* subsp. *carotovora*) are important bacterial pathogen to cause decay during storage, which affect the tuber germination and leads to appearance of disease in subsequent crop, when it is used as seed.

In this study, we developed PCR assay for specific detection and identification of *E. carotovora* subsp. *carotovora* most frequently associated with potato, causing soft rot disease in potato. PCR protocol was standardized to detect *E. carotovora* subsp. *carotovora* not only in infected/rotted potato tubers but also from very low bacterial inoculums in asymptomatic, latently infected potato tubers.

The detection limit of the PCR assay was 1.0 ng DNA for *E. carotovora* subsp. *carotovora*. Assays on CFU detection, allowed an average detection of 10^3 cfu/ ml on *E. carotovora* subsp. *carotovora*.

The pathogen detection in PCR assay limited in several ways due to the presence of competitor DNA matrix and the length of the amplified PCR products (Markoulatos *et al*., 2002) [16]. The primer designed for detection of *E. carotovora* subsp. *carotovora* amplify a single copy gene, *i.e.,* pectatelyase gene. The developed PCR assay demonstrated high specificity for detection of *E. carotovora* subsp. *carotovora*. There is no false results observed for bacteria that can possibly co-exist with the pectinolytic ones in potato ecosystem and / or belonging to the species *Chryseobacterium*, *Paenibacillus*, *Flavobacterium*, *Pseudomonas* and *Xanthomonas* that according to the literature (Mikicinski *et al*., 2010a and b) [17, 18]. Fraaije *et al.,* 2008 [10], evaluated the sensitivity of the PCR for a direct detection of *Eca* in crude peel extracts was only 10^7-10^8 cells/ml, due to inhibition of PCR amplification by potato tuber-derived compounds. The 16S-23S rRNA intergenic transcribed spacer (ITS) was used for identification the soft rot *Erwinias*. The ITS was amplified from *Erwinia* and other genera using universal PCR primers (Toth *et al.,* 2001) [12].

The PCR assay allows much faster and at the same time more reliable detection than conventional methods used for *E. carotovora* subsp. *carotovora* monitoring. Plating the suspected plant extracts and the analyses with standard PCR procedures require couple of days to complete and do not always result in isolation and characterisation of the causative agents (Perombelon and van der Wolf, 2002) [22].

There is urgent need to prevent postharvest losses due to spoilage and deterioration of quality of potato tubers during storage and also check the spread of bacterial pathogens through stored potato and rotting of potato by *Erwinia carotovora* subsp. *carotovora* during storage and their subsequent transmission in next crop to cause disease in the field. Therefore, there is an urgent need to develop a specific and sensitive polymerase chain reaction (PCR) assay for the rapid, accurate detection of *E. carotovora* subsp. *carotovora* in asymptomatic potato tuber/plant samples, which will be helpful in seed certification programme to make sure that tuber free from this deadly pathogen.

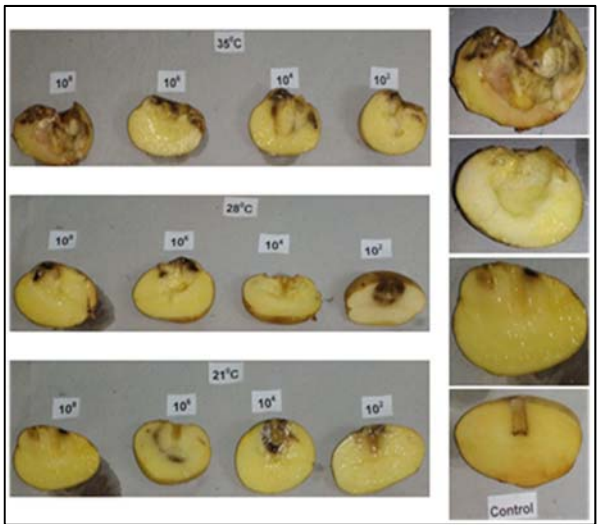

Fig 1: Rot of potato tuber by artificial inoculation of *E. carotovora* subsp. *carotovora* at different Population level and incubation temperature

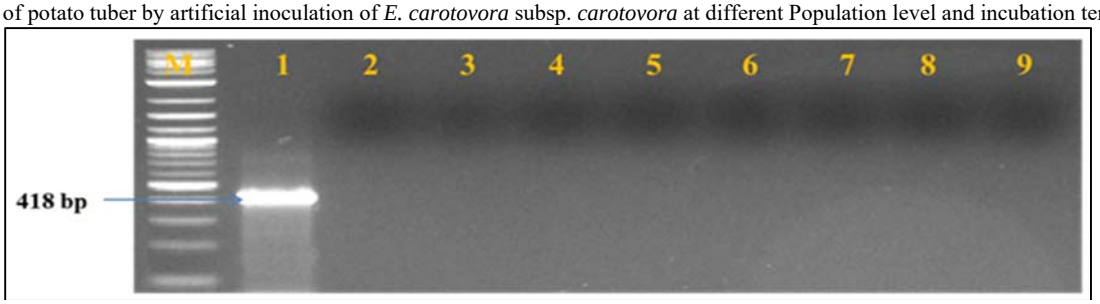

Fig 2: Specificity test of desined primer EF/ER, amplify at 418 bp for detection of *Erwinia carotovora* ssp. *carotovora*. Lanes 1- *Erwinia carotovora* ssp. *Carotovora*, 2- *Ralstonia solanacearum*, 3-*Pantoea agglomerans*, 4-*Pseudomonas fluorescens*, 5-*Xanthomonas campestris* pv. *oryzae*, 6- *Xanthomonas campestris* pv. *punicae*, 7- *Xanthomonas campestris* pv. *campestris*, 8- *Bacillus subtilis*, 9- Negative control. Lane M = 100 bp DNA Ladder.

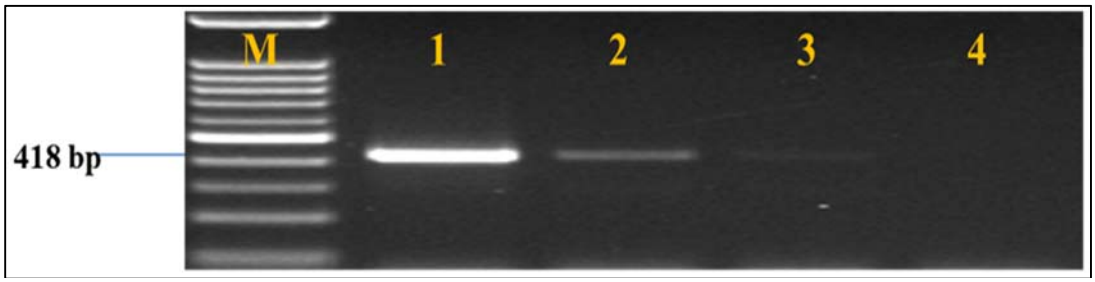

Fig 3: Detection limits of *E. carotovora ssp. carotovora* tempelate concentration lanes 1 (100ng), 2 (10 ng), 3 (1 ng) and 4 (.1 ng) in PCR assay with EF/ER primers amplify at 418 bp. Lane M = 100 bp DNA ladder.

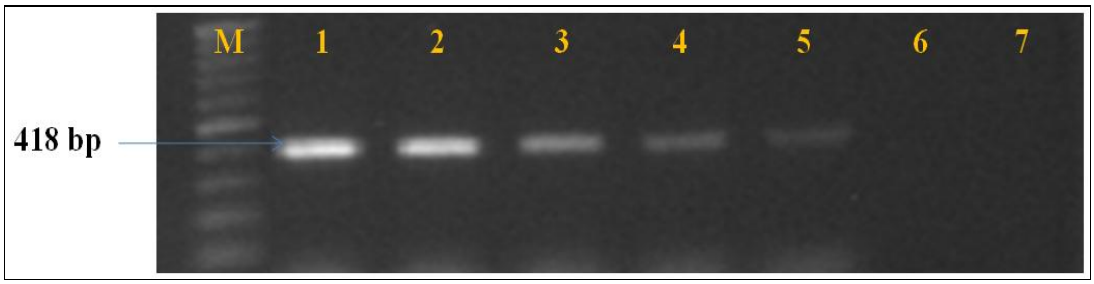

Fig 4: Detection limits of *E. carotovora* subsp. *carotovora* at different level of serial dilution in PCR assay by EF/ER primers amplify at 418 bp. Lanes 1 .10^7cfuiml, 2:10^6cfulml, 3 :10^5cfulml, 4: 10^4 cfulml, 5: 10^3cfulml, 6: 10^2cfulml, 7: 10^1 cfu/ml. Lane M: 100 by DNA ladder.

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