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PHYSIOLOGY OF ERWINIAS ASSOCIATED WITH BLACK LEG OF POTATO

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Studies on some of the physiological aspects of the bacterium *Erwinia carotovora* were carried out, which revealed that the optimum temperature for the bacterial growth was found to be 35 ^{o}C. *Erwinia carotovora* grew best in slightly alkaline pH at the pH of 7 and 8. Maximum bacterial growth was found on the nutrient agar medium containing potassium nitrate as a nitrogen source, while the nutrient agar medium amended with glucose was found to be the most suitable for bacterial growth.
Keywords: Physiology, Erwinias carotovora, sub sp. atroseptica, pH, temperature, carbon sources, nitrogen sources

INTRODUCTION

Blackleg and soft rot are commonly occurring disease in field as well as during transit and storage. Due to its endemic nature, blackleg disease caused by *Erwinia c. atroseptica* is prevalent in cool and temperate regions of Canada, the US, Western Europe India and Pakistan (Molina and Harrison, 1977 Caron *et al*, 1979; Persson 1988; Bain *et al*, 1990, Hafiz, 1986). *Erwinia carotovora* subsp. *atroseptica* (Eca) and *Erwinia carotovora* subsp. *carotovora* (Ecc) are considered the main source of primary inoculum for blackleg and soft rot of potato. They are responsible for losses both quantitatively and qualitatively. Both subspecies are commonly associated with potato tuber soft rot, but Eca usually causes rot in the basal part of the stem (blackleg disease). *Erwinia carotovora* subsp. *atroseptica* occurs in both temperate and warm climates but mostly in storage. The rotting of mother tubers during the growing season has been reported as the major source of inoculum for contaminating progeny tubers, which later in storage, when conditions are favourable, could lead to losses due to soft rot of tubers (Perombelon, 1992).

Jones (1901) isolated and described for the first time the soft rot bacterium as *Bacillus carotovorus*. Hingorani and Addy (1953) investigated the blackleg disease of potato in India with special reference to the properties of the causal organism. El-Goorani and El-Kazzaz (1975) have reported decrease in germination of potato tubers in soil infested with soft rot causing bacterium.

On the basis of field symptoms, the disease was reported for first time in Pakistan in 1984 from Swat valley (Khan, *et al.,* 1985). From hilly areas and plains of Punjab, Turkensteen (1986 and 1987) reported it in 1985 and 1986, respectively. The disease was more frequent in the districts of Sialkot, Gujranwala and Faisalabad with an incidence ranging between 0.2-2.9% in Desiree, Ultimus, Multa, Patrons and Cardinal varieties (Hafiz, 1986). Keeping in view, the importance of the disease and speculated losses, it was thought essential to investigate some of the physiological aspects of *Erwinia carotovora*, associated with blackleg disease of potato, in order to get an insight of the problem.

MATERIAL AND METHODS

Isolation of the pathogen associated with blackleg disease of potato

Potato (*Solanum tuberosum* L.) plants showing typical symptoms of blackleg were collected in polythene bags and brought to laboratory for isolation of bacterium. For the isolation of the pathogen, infected portion of the stem blackened at collar region were cut into small pieces and disinfected by dipping in 0.5% Mercuric Chloride ($HgCl_2$) to reduce surface mycoflora. Then two washings were given with distilled water to reduce the injurious effects of $HgCl_2$, before drying by placing on filter paper.

The disinfected bits of potato plants from diseased portions were then plated on solidified nutrient agar medium (Khan *et al.,* 1999) in Petri plates and incubated at 30 ± 2 ^{o}C. Grayish-white bacterial growth appeared around the cutted stem tissues of the diseased plant.

Purification and multiplication of pathogen

Sterilized streaking loop was touched on bacterial growth and then streaked in the Petri plates containing media in criss cross manner to obtain well-separated bacterial colonies. Isolated bacterial colonies were picked and each colony was transferred to separate Nutrient agar slants.

On the basis of characteristics described by Bergey (1923) the bacterium was identified as *Erwinia carotovora*.

Physiological studies

Effect of some of the physiological aspects i.e; temperatures, pH levels, nitrogen and carbon sources on the growth of *Erwinia carotovora*, associated with blackleg disease of potato were investigated. In all experiments twenty-mL (20 mL) of the culture medium were poured in to the Petri plates (9 cm) in triplicates.

The autoclaved nutrient agar media were inoculated with bacteria inoculum by placing it in the centre of Petri plates aseptically and incubated at 35 ± 2 °C. The bacterial growth was recorded by measuring the colony diameter along two axes at right angle to each other after every 24 hours of incubation for four days.

Temperature

Four different temperatures 20, 25, 30 and 35 ± 2 °C were used to assess their suitability for the growth of *Erwinia carotovora* on nutrient agar medium. The colony growth was recorded after each 24 hours of incubation for four days.

pH

The effect of various pH levels on the bacterial growth were studied using Nutrient agar medium as the substrate. A quantity of 250 mL of nutrient agar medium taken in flask was adjusted to pH 5, 6, 7 and 8 by the addition of appropriate volume of 1.0 N HCl or NaOH solutions. The autoclaved, solidified medium was inoculated with bacterium and incubated at 35 ± 2 °C.. The data for bacterial growth was recorded after every 24 hours.

Nitrogen sources

The amount of nitrogen which was provided through 5 g peptone, was replaced by 5 g potassium nitrate (KNO_3), 2 g ammonium nitrate (NH_4NO_3) and 1.5 g urea. These nitrogen sources were added in nutrient agar medium by preparing their solutions before autoclaving. The inoculated Petri plates were incubated at 35 ± 2 °C. The diameter of the bacterial colony was recorded after 24, 48, 72 and 96 hours of incubation.

Carbon sources

The ingredients required for the preparation of 250 ml nutrient agar medium were mixed in 220 mL distilled water. In the remaining 30 mL distilled water, 3 g of different carbon sources i.e., glucose, sucrose, fructose and maltose were mixed in separate flasks. After that, these sugar solutions were sterilized by filtration and tyndelyzation in separate sterilized flasks (Awan and Sajjad, 2005). These sterilized sugar solutions were mixed in autoclaved medium, before solidification. The inoculated Petri plates were incubated at 35 ± 2 °C. The diameter of the bacterial colonies were recorded after the intervals of 24 hours.
The data recorded on the colony diameter of *Erwinia carotovora* was statically analyzed and the means were calculated at 5 % level of significance (Steel *et al.,* 1996).

RESULTS

Temperature effects

Bacteria are unable to exhibit efficient growth in the laboratory unless they are incubated at proper temperature. In order to determine which temperature is best for the growth of *Erwinia carotovora*, a range of 20 – 35 ± 2 °C was employed, with a difference of 5 °C, on Nutrient agar medium. The bacterial growth was observed at all temperature, but the best results were obtained at 35 ± 2 °C as the bacterial growth at this temperature was 9 cm after 96 hours of incubation (Fig. 1).

Fig. 1. Effect of different temperatures on the in vitro growth of *Erwinia carotovora* at various incubation periods.

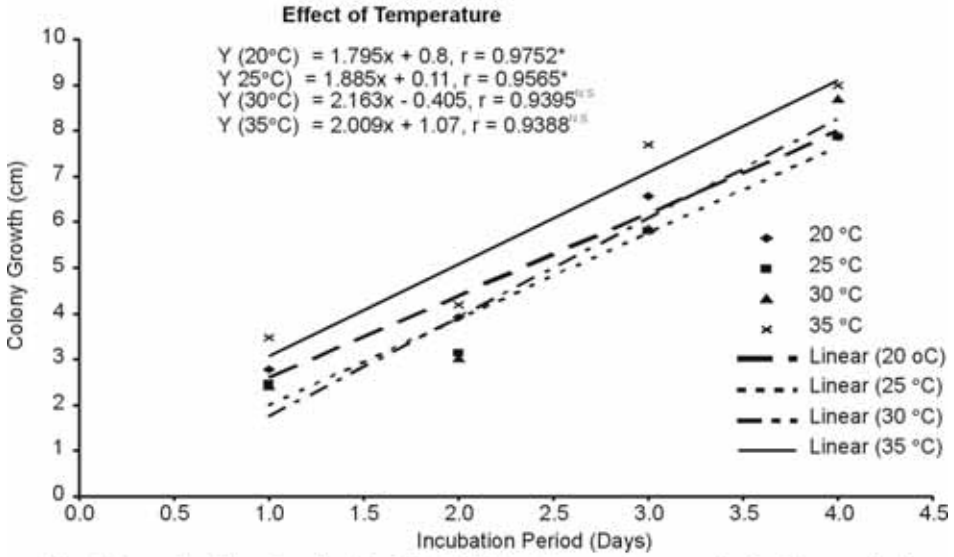

Fig.1. Regression lines showing the effect of different temperatures on the in vitro growth of *Erwinia carotovora* at various incubation periods.

The rate of increase in colony growth was significant at 20 and 25 ^{o}C (P = 0.05). However, at 30 and 35 ^{o}C the colony growth was non significant (Fig.1).

pH effects

One of the important factors that affect growth of bacteria and many other life processes is the hydrogen ion concentration (pH) of the growth medium. To asses this various pH values ranging from 5–8, with the difference of 1 were evaluated using nutrient agar medium at 35 ± 2 ^{o}C. When the effects of pH levels on bacterial growth was compared after 96 hours of incubation, the pH 7 and 8 was found to be the most suitable for bacterial growth as on these pH levels maximum growth 8.70 cm and 9.00 cm was obtained. The rate of increase in colony growth was significant at pH 5 and 7 (P = 0.05) while at pH 6 and 8 the colony growth was non significant (Fig 2).

for bacterial growth as on it maximum growth 8.17 cm was obtained. The rate of increase in colony growth was significant on the nutrient agar medium amended with potassium nitrate, urea and peptone (P = 0.01). The colony growth was also significant on the nutrient agar medium amended with ammonium nitrate (P = 0.05) (Fig 3).

Carbon sources effects

After 24 hours of incubation of *Erwinia carotovora*, the bacterial growth on the medium having sucrose was greater. However, with the passage of time i.e; after 96 hours of incubation, the nutrient agar medium amended with glucose was found to be the most suitable for bacterial growth as on it maximum growth 9.00 cm was obtained. The rate of increase in colony growth was significant on the nutrient agar medium amended with maltose (P = 0.05). However, the colony

Fig. 2. Effect of different pH levels on the in vitro growth of *Erwinia carotovora* at various incubation periods.

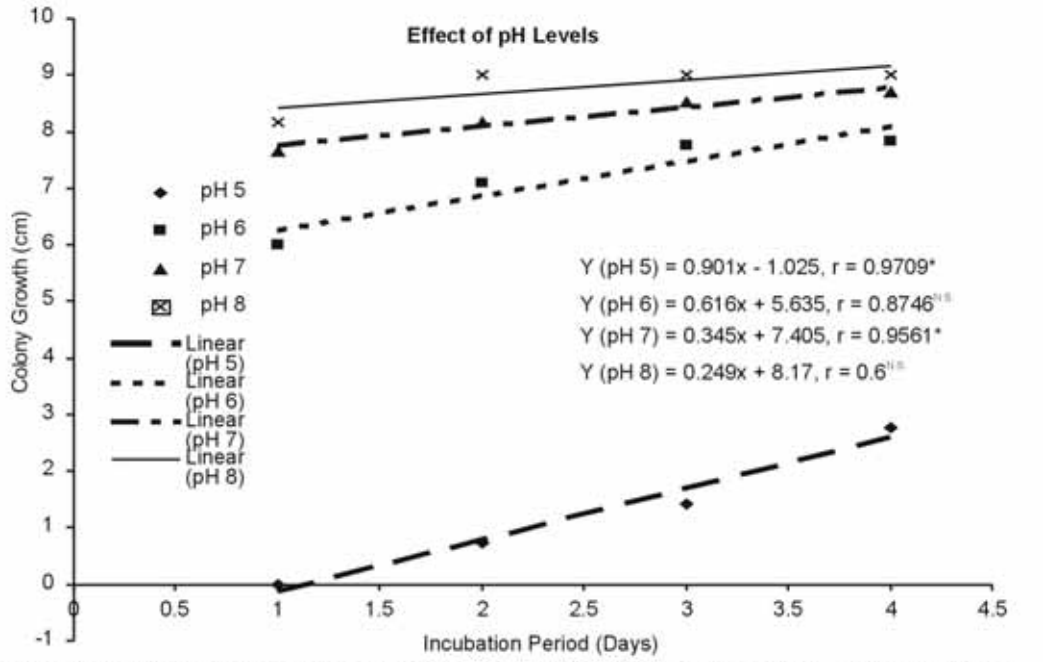

Fig. 2. Regression lines showing the effect of different pH levels on the in vitro growth of *Erwinia carotovora* at various incubation periods.

Nitrogen sources effects

In case of nitrogen requirement after 24 hours of incubation of *Erwinia carotovora* inoculated on nutrient agar medium, which was amended by ammonium nitrate, the colony growth was greater as compared to others. However, when the effects of different nitrogen sources on bacterial growth was compared at 96 hours of incubation, the bacterial growth in potassium nitrate amended petriplates was found to be the most suitable

growth was non significant on the nutrient agar medium amended with sucrose, fructose and glucose (Fig. 4).

DISCUSSION

Physiology in its broadest sense phenomena exhibited by living organisms and it is description of certain essential properties and behavior of organisms in chemical and physical term. The nutrition of bacteria is

Fig. 3. Effect of different nitrogen sources on the in vitro growth of *Erwinia carotovora* at various incubation periods.

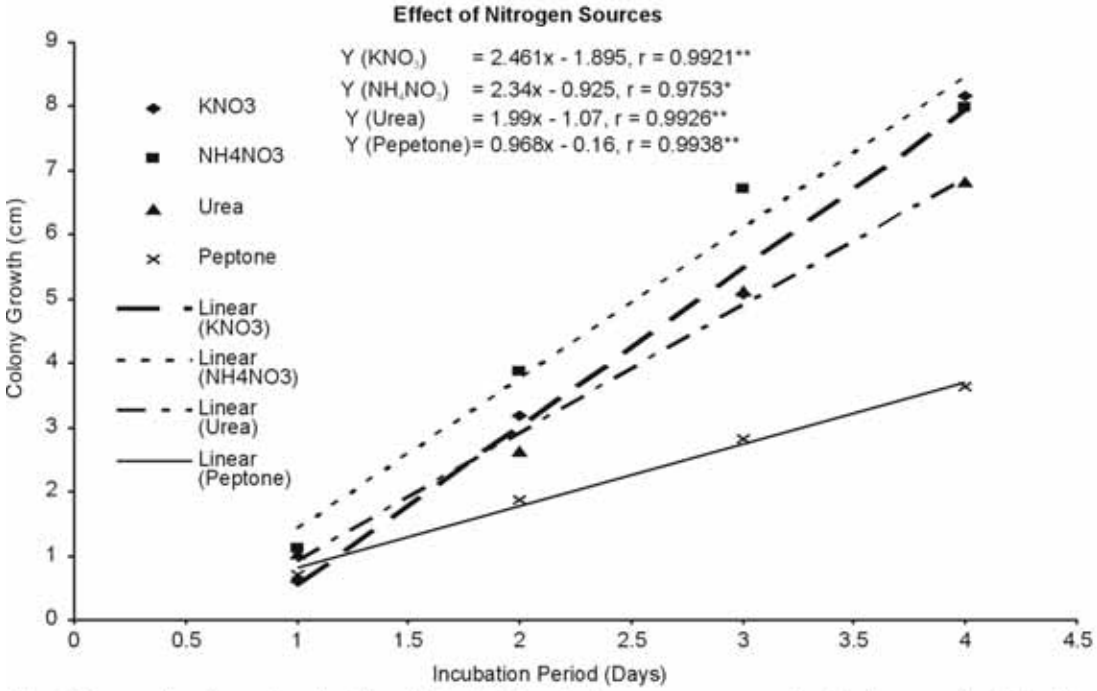

Fig. 3. Regression lines showing the effect of different nitrogen sources on the in vitro growth of *Erwinia carotovora* at various incubation periods.

very important for studying its physiology (Goto, 1991). *Erwinia carotovora* that is the causal organism of the black leg of potato (Pe´rombelon and Kelman 1980; Pe´rombelon 1992) was studied *in vitro* to find its response to various nutrient sources. It can be differentiated from all other *E. carotovora* strains solely on the basis of acid production from *α*-methylglucoside, production of reducing substances from sucrose, and inability to grow at 37°C (Graham, 1972; De.Boer *et al*). Along with the availability of food source or substrate, bacteria are unable to exhibit efficient growth in the laboratory unless they incubated at the temperature optimum for their growth. Bacteria differ greatly for optimum temperature for their growth. The range of temperature best for the growth of *Erwinia carotovora* is 20-35 ^{o}C with a difference of 5^{o}C on the nutrient agar media. The occurrence of the disease and the damage caused by the disease is temperature dependent firstly the bacteria actively multiplied at the infection sites and than it produce numerous extra cellular polysaccharides (Smadja *et al*, 2004 Malcolmson, 1959) .The bacterial growth at each temperature increased with increased incubation period. After 24 hours of incubation period, the bacterial growth was statistically same at 20, 25 and 30^{o}C. However, at temperature of 35^{o}C the bacterial growth is greater as compared to others, which was found to be the optimal temperature for bacterial growth, which was not the same for all the species of bacteria few isolates can grow at temperature of 32^{o}C but a few can grow at 35^{o}C and none can grow at 37^{o}C the thermal death point of the bacteria is 48^{o}C on the other hand *E.carotovora spp carotovora* can grow at temperature of 38^{o}C (Malcolmson, 1959) On the basis of different physiological and biochemical characteristics Eca and Ecc can be differentiate (Perombelon & Kelman, 1980,. Jabuonski *et al* 1986). However there are some research report which indicate that Ecc don't have the ability to grow at 37^{o}C (Hyman *et al*, 1998, Lelliot & Dickey 1984) According to Singh (1998) 27 to 30 ^{o}C was the optimum temperature for growth of Erwinia. However, the maximum temperature for bacterial growth varies with isolates. Ecc has a maximum temperature for growth at 37 - 40 ^{o}C while Eca does not grow at temperatures above 35 ^{o}C.

On of the important factors, responsible for the bacterial growth is the hydrogen ion concentration (pH) of the medium upon which the bacteria grows. The pH of the medium exerts effect upon the rate and the amount of growth and many other life processes of bacteria. The suitable pH value on nutrient agar

Fig. 4. Effect of different Carbon sources on the in vitro growth of *Erwinia carotovora* at various incubation periods.

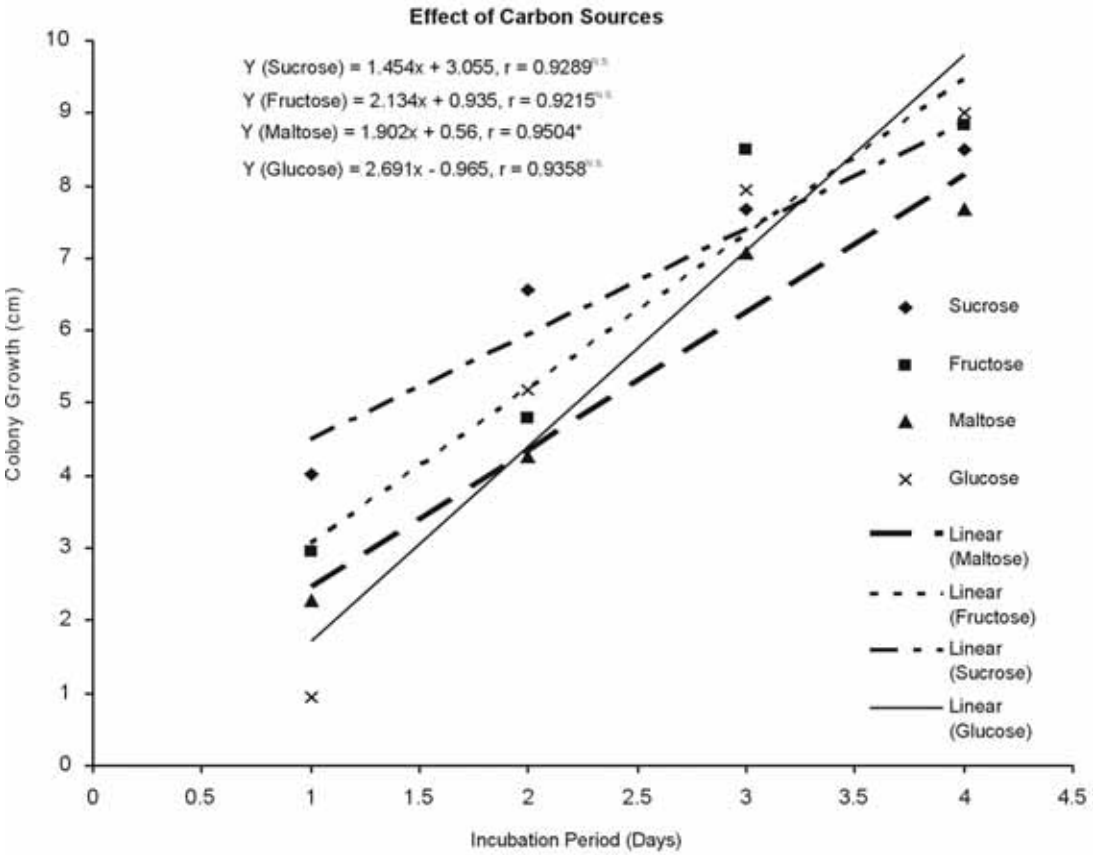

Fig. 4. Regression lines showing the effect of different Carbon sources on the in vitro growth of *Erwinia carotovora* at various incubation periods.

medium for maximum growth of *Erwinia carotovora* indicates that *Erwinia carotovora* preferred a slightly alkaline pH. The pH 7 and 8 was found to be the most suitable for bacterial growth as on these pH levels maximum growth 8.7 cm and 9.0 cm was obtained. The pH range for optimal growth was between 7.0 and 8.0, and the growth was maximal at pH 7.5. No bacterial growth occurred either below pH 4.0 or above pH 10.5. Based on this result, the effect of the highly acidic or alkaline salts (which strongly affected the pH of the medium) on the growth of *E.c. atroseptica* was evaluated at pH 7.5. (Elian *et al* 2005).

Bacteria also require nutrients from the environment for various activities such as the synthesis of their cell material and for generation of energy. Nitrogen and carbon are among the major essential elements for bacterial growth. Different bacteria use different sources of nitrogen and carbon efficiently. Bacteria for carrying out different metabolic and structural functions use nitrogen from different sources. The suitable sources of nitrogen for *Erwinia carotovora* having four different nitrogen sources (i.e; potassium nitrate, ammonium nitrate, urea and peptone) amended nutrient agar medium were used for the study of bacterial growth. *Erwinia carotovora* grew best on the nutrient agar medium containing potassium nitrate as on it maximum growth 8.17 cm was obtained after 96 hours of incubation. Other nitrogen sources also supported the growth of bacteria (Goto, 1991, Malcolmson, 1959).

It is important to find the qualitative and quantitative requirements of plant pathogens. The bacteria may utilize certain simple form or may convert complex carbon compounds into simple form, which may be readily metabolized. There is difference on the utilization of different carbon sources in the literature Eca can utilized maltose as carbon source while Ecc is unable to utilized maltose as sole source of carbon (Malcolmson, 1959). There is also conflicting reports for the sole carbon utilization as (Duarte *et al* 2004)

find that strains of BPBB did not utilized Tween-40 nor Tween–80 as sole carbon source. So in our study the carbon source utilized by the bacterium is glucose as the nutrient agar medium amended with glucose was found to be the most suitable for bacterial growth as on it maximum growth 9.00 cm was obtained after 96 hours of incubation. The utilization of different carbon sources and inability of the bacterium at 37^{o}C is also important for the identification of different subspecies of Erwinas (Zaidi *et al*., 2003).

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