INCIDENCE OF ERWINIA CAROTOVORA WITHIN MANITOBA POTATOES AND THE EFFECT OF LOW TEMPERATURES ON THE IN VITRO GROWTH AND SOIL SURVIVAL OF THE BACTERIA

BY

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ABSTRACT

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Incidence of Erwinia carotovora within Manitoba potatoes and the effect of low temperatures on the in vitro growth and soil survival of the bacteria.

Major Professor; Claude C. Bernier.

Manitoba's seed potato program is based on increasing stock by stem cuttings and involves the regular testing of these cuttings for the blackleg organism, Erwinia carotovora variety atroseptica and the related soft rot organism, Erwinia carotovora variety carotovora. Testing began in the spring of 1976 and continued in 1977 and 1978. Some mother tubers were contaminated with both organisms in the first two years of testing, but in 1978, all mother tubers appeared free from infection. In addition, a survey of Manitoba's commercial stock was conducted during the fall and winter of 1977. The results of this study indicated that 59% of the sampled tubers rotted due to soft rot contamination. E. carotovora var. carotovora was recovered more frequently than E. carotovora var. atroseptica in the rotting tubers.

A series of five experiments was conducted to study the ability of E. carotovora var. carotovora and E. carotovora var. atroseptica to survive in soil under controlled and natural environments. Several

procedures of soil inoculation and bacterial recovery were evaluated. The results indicated that low temperatures and association with plant debris prolonged the survival of E. carotovora in soil. Both varieties of E. carotovora remained viable in soil over winter under Manitoba climatic conditions and could possibly serve as a source of inoculum in the spring.

The in vitro growth of E. carotovora was studied at temperatures of 10°C, 15°C and 20°C. There were no significant differences in growth rate between E. carotovora var. carotovora and E. carotovora var. atroseptica at the latter two temperatures. At 10°C isolates of E. carotovora var. atroseptica grew significantly faster compared to isolates of E. carotovora var. carotovora. The greatest variation among isolates was observed at the lowest temperature.

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INTRODUCTION

Blackleg and soft rot, caused by Erwinia carotovora (Jones) Bergey et al. variety atroseptica (van Hall) Dye and Erwinia carotovora (Jones) Bergey et al. variety carotovora (Jones) Dye, respectively, are important bacterial diseases of potatoes in Manitoba. Previous researchers (Graham and Dowson, 1960; Lazur and Bucur, 1964; Logan, 1968; Graham and Hardie, 1971; Pérombelon, 1971) have identified E. carotovora var. atroseptica as the sole cause of blackleg. Recent reports by Stanghellini and Meneley (1975) and Molina and Harrison (1977) have indicated that E. carotovora var. carotovora can also cause blackleg infections in the field. For this reason, it is important to identify the causal agent of blackleg in different areas and to study the relationship of both organisms.

An understanding of the occurrence and duration of a soil-borne phase of E. carotovora is basic to developing controls for blackleg and soft rot. The production of blackleg-free seed potatoes would be a logical control method provided the bacteria are not present in planting soils.

The major objective of this study was to determine the extent of contamination of E. carotovora var. atroseptica and E. carotovora var. carotovora in Manitoba potatoes and to study the effect of low temperatures on the growth and soil survival of the bacteria.

REVIEW OF LITERATURE

Introduction:

Soft rot and blackleg of potato occur worldwide and cause considerable losses in the field, in transit and in storage (Bonde, 1950; Agrios, 1969; Roberts and Boothroyd, 1975). E. carotovora var. carotovora is the causal agent of soft rot and E. carotovora var. atroseptica causes blackleg and a storage rot of potato tubers. Soft rot and blackleg have similar disease characteristics due to the close relationship of the causal agents. However, it is well established that there are enough differences detween the organisms to warrant their classification as separate varieties of a single species, E. carotovora (Dye, 1969). For these reasons it is important to gain information about the soft rot-blackleg disease complex and an understanding of the relationship of the causal organisms.

Seed Potato Certification Programs:

Potato seed certification began in Europe in the twentieth century primarily for the control of virus diseases. The programs have been expanded gradually to include tuber-borne diseases caused by other pathogens. At present, a very extensive and long-lived certification program remains in operation to regulate and control potato seed quality.

The highest levels of purity and health in potato crops are achieved through growers' participation in the seed potato program operated by the Canada Department of Agriculture. The main feature of

this program is the production and maintenance of elite classes of seed (Hodgson et al., 1974). There are five classes of seed potatoes in Canada. Ranked from highest to lowest in seed quality the classes are Elite I, Elite II, Elite III, Foundation and Certified.

The Canada Department of Agriculture is responsible for setting minimum necessary standards with respect to specific diseases and to a lesser extent other qualifications such as varietal mixtures. The basic principles followed in formulating regulations have been described by Leach (1938) as follows: 1) an accurate knowledge of the extent to which a disease is transmitted in or on seed tubers, 2) the recognition of all other sources of infection and 3) an accurate evaluation of different sources of infection under various circumstances. Using this information the Canada Department of Agriculture has determined tolerances for each disease monitored within the program. Presently, seed potatoes in Manitoba are monitored for blackleg, Fusarium wilt, ring rot, leaf roll, mosaic and spindle tuber.

Disease detection and diagnoses are accomplished by two or three field inspections performed by Canada Department of Agriculture personnel during the growing season. Aside from diseases with zero tolerance, tolerance differentials between the first and last inspection permit growers to rogue out infected plants between inspections. If tolerances are exceeded at the final reading, the crop is not eligible for certification.

Non-latent viral, bacterial, fungal and mycoplasmal diseases are nearly always diagnosed on the basis of symptoms visible in the field. Field diagnoses often are difficult, since symptom expression frequently is influenced by the environment and is subject to differences in varietal

response to certain pathogens. Occasionally, laboratory diagnostic techniques are employed to assist in some decisions. Such techniques must furnish accurate results quickly as the time interval between inspection and harvest is usually insufficient for the complete application of Koch's postulates.

Blackleg causes a serious problem for seed growers due to stricter tolerance levels accepted into the certification programs in recent years (Pérombelon, 1972a). The reason for this is that tuber lenticels of many North American (De Boer and Kelman, 1975 and European (Pérombelon, 1972a) potato varieties are universally infected but the conditions necessary for the expression of blackleg symptoms in the field are poorly understood (Pérombelon, 1972a). Consequently, certification programs are beginning to incorporate tests for the presence of the bacterium instead of relying on visual field symptoms. In Manitoba, many seed potato fields have been rejected due to blackleg disease between 1971 and 1977. The data presented in Table 1 indicate that blackleg is a major factor in the rejection of seed potato fields.

Disease Symptoms and Cycle:

Symptoms of soft rot and blackleg tuber infection appear as water soaked lesions of the lenticels. The lesions gradually enlarge due to the multiplication of the bacteria and the increased activity of pectolytic enzymes. Then, a slimy mass of disorganized cells exudes to the surface as cracks develop in the tuber.

The characteristic symptom of blackleg disease appears as a blackening and soft rot of the basal part of affected plants. Foliar symptoms appear at flowering and consist of a rolling and chlorosis of

TABLE 1. Summary of rejections of seed potato fields inspected in Manitoba from 1971 to 1977.

CAUSE OF REJECTION	YEAR							
	1971	1972	1973	1974	1975	1976	1977	Total
Wilts		1						1
Ring rot	3	1		4	4	9		21
Ring rot(farm)[a]	8	3		28	24			63
Blackleg	6	2	1			1	3	13
Leaf roll	3			2	9	1		15
Spindle tuber				2	2	14		18
Foreign			1					1
Weeds						3		3
Miscellaneous	SP[b]	1[c]						2
Total	21	8	2	36	39	28	3	137

[a] Fields rejected due to ring rot on the same farm.

[b] SP = seed plot. Rejected due to mixture with Norchip.

[c] Rejected due to mixture with a red variety.

the upper leaves. Infected plants wilt and die as the lower stems are girdled with rot (Bonde, 1950). These symptoms are not characteristic of soft rot disease and in this regard blackleg differs from soft rot.

Many bacterial cells are released into the soil from the rotting seed piece (De Boer et al., 1975) and can contaminate newly formed daughter tubers (Graham and Harper, 1967; De Boer et al., 1974). Tuber infections can also result from bacterial transmission from infected to healthy plants growing a few feet apart (Graham and Harper, 1967; Pérombelon and Lowe, 1971).

The bacteria have been reported to survive for many months in infected plant debris (Logan, 1969; Ficke et al., 1973; Burr and Schroth, 1977), in stored potato tubers (Bonde, 1950; Lazur and Bucur, 1964; Pérombelon, 1972; De Boer and Kelman, 1975; Vruggink and Maas Geesteranus, 1975), and in the soil (Ficke et al., 1973; Meneley and Stanghellini, 1976; Mew et al., 1976; Burr and Schroth, 1977).

Blackleg is a bacterial disease peculiar to the potato (Bonde, 1950). Soft rot occurs on numerous vegetable and ornamental crops and has been reported on the following: delphinium, onions, carrots, leeks, cabbage, celery, asparagus, beets, kohlrabi, caladium, red pepper, canna, cucumber, lettuce, muskmelon, cyclamen, dahlia, gladiolus, hyacinth, iris, tomato, tobacco, parsnip, radish, rhubarb, potato, eggplant, squash, spinach and calla lily (Conners, 1967; Agrios, 1969). Infection of the tubers, roots, bulbs, succulent stems, stalks and leaves can occur, resulting in similar disease symptoms on all hosts. Blackleg and soft rot are favoured by cool, wet soil (Van den Boom, 1967; Pérombelon, 1976) and by continuous cropping of potatoes.

Taxonomic classification and description:

Jones (1901) isolated a bacterium capable of producing a soft rot of many fleshy vegetables. He named the causal agent Bacillus carotovorus. Van Hall (1902) attributed blackleg and soft rot of potato to Bacillus atrosepticus. For many years Bacillus carotovorus and Bacillus atrosepticus were thought to be different species. Dye (1969) has conducted an extensive study of both species and observed that Bacillus carotovorus and Bacillus atrosepticus were similar in morphology and biochemical behaviour. He concluded that the two species were closely related and proposed that they be considered as varieties of a single species, Erwinia carotovora. This proposal is in agreement with Bergey's Manual of Determinative Bacteriology (1974). The currently accepted classification of these organisms is Erwinia carotovora (Jones) Bergey et al. variety atroseptica (van Hall) Dye and Erwinia carotovora (Jones) Bergey et al. variety carotovora (Jones) Dye.

Erwinia carotovora cells are predominantly single, straight rods 0.5 - 1.0 by 1.0 - 3.0 microns and are motile by peritrichous flagella (Bergey, 1974). The cells are gram negative, oxidase negative, catalase positive and facultative anaerobes in glucose deep stabs (Elliot, 1951; Dye, 1969; Bergey, 1974). Erwinia carotovora forms white, round, raised, smooth and glistening colonies on nutrient agar (Elliott, 1951).

Identification:

E. carotovora secretes pectolytic enzymes which destroy the middle lamella of the cell walls of plant tissue and, in vitro, will liquefy pectate gel (Stewart, 1962; Logan, 1963). This property has been utilized by many researchers (Stewart, 1962; Logan, 1963; Miller and

Schroth, 1970; Cuppels and Kelman, 1974) in the preparation of selective media for the isolation of E. carotovora.

Cuppels and Kelman (1974) have evaluated many of these selective media. They found that crystal violet pectate medium was the most efficient in recovering E. carotovora from soil and plant material. The crystal violet pectate medium differentiated pectolytic and non-pectolytic bacteria and suppressed the growth of gram positive bacteria. E. carotovora could be recognized easily by colony morphology and the type of depression formed on the plates. Erwinia colonies appeared iridescent, translucent, and crisscrossed with internal markings when examined under oblique light. Secondly, Erwinia colonies produced deep, cup-like depressions as compared to the wide, shallow depressions formed by other pectolytic soil bacteria.

The type of soil and the number of soft rot bacteria present in the soil may affect the efficiency of recovering E. carotovora from field soil using crystal violet pectate medium. For this reason, Cuppels and Kelman (1974) reported that percent recovery of E. carotovora varied between 65 and 100. In addition, they stated that crystal violet pectate medium would be unsuitable for isolating low populations of E. carotovora from soil. Inoculum concentrations of 10^2 cells per gram of soil were not easily recovered due to the rapid growth of other soil bacteria.

Erwinia colonies are readily visible on crystal violet pectate medium after a 48-72 hour incubation period at 25°C. Then, E. carotovora can be identified by the following criteria: 1) ability to ferment glucose within 48-72 hours, 2) gram-negative, 3) oxidase negative, and 4) inability to fluoresce on Kings' Medium B (Dye, 1969; Burr and Schroth,

1977).

Varietal differentiation may be determined by the following series of tests: 1) ability to grow at 36°C, 2) ability to produce reducing sugars from sucrose, 3) ability to produce acid from ∝-methyl glucoside and 4) precipitation with atroseptica antiserum (Dye, 1969; Graham, 1972; De Boer and Kelman, 1975; Burr and Schroth, 1977). The majority of E. carotovora var. carotovora isolates are capable of growing at 36°C but produce negative reactions to all other above-mentioned tests. E. carotovora var. atroseptica isolates can be identified by their ability to produce positive reactions to all of the aforementioned tests except that of growth at 36°C.

Many researchers (Stanghellini and Meneley, 1975; Meneley and Stanghellini, 1976; Burr and Schroth, 1977) have reported that some isolates are difficult to classify as either variety by observing their reactions to the aforementioned tests. These isolates are grouped in an atypical category which includes E. carotovora var. carotovora and/or E. carotovora var. atroseptica.

Recently, serology has been employed to detect E. carotovora var. atroseptica in plant tissue (Graham, 1965; Stanghellini et al., 1967; Vruggink and Maas Geesteranus, 1975; Allen and Kelman, 1977). The two methods routinely used are gel diffusion and slide agglutination. In both methods plant sap is mixed with atroseptica antiserum and presence of E. carotovora var. atroseptica in the plant sap is indicated by a flocculent precipitate. The gel diffusion method is the more specific of the two tests especially if absorbed atroseptica antiserum is used (Graham, 1963; Vruggink and Maas Geesteranus, 1975).

Serology is faster and more efficient compared with selective media and biochemical tests for identifying E. carotovora var. atroseptica (Vruggink and Maas Geesteranus, 1975). It is especially practical in seed certification programs where large numbers of potato stems and tubers are tested (Graham, 1963; Vruggink and Maas Geesteranus, 1975).

There has been a continuing effort to detect significant differences in pathogenic characteristics between E. carotovora var. carotovora and E. carotovora var. atroseptica. Burkholder and Smith (1949) and Smith (1950) reported that they could differentiate among soft rot Erwinia isolates obtained from various hosts. E. carotovora isolated from potato could readily produce blackleg symptoms when inoculated into young potato plants, whereas the majority of isolates obtained from other hosts could not produce stem blackening.

Rudd Jones (1950) and Hellmers and Dowson (1953) conducted similar experiments and their results did not agree with earlier research. In both studies, the researchers were able to produce blackleg symptoms with all E. carotovora isolates provided the organism was introduced directly into the vascular tissue of potato stems.

Erinle (1975) reported that site of inoculation was a major factor in differentiating between E. carotovora var. carotovora and E. carotovora var. atroseptica. The two inoculation sites evaluated in his study were seed tuber and lower stem. Isolates of E. carotovora var. atroseptica produced blackleg symptoms with every inoculation whereas E. carotovora var. carotovora produced disease symptoms with lower stem inoculation only. The failure of E. carotovora var. carotovora to produce blackleg with seed tuber inoculation demonstrated that this was a more reliable method of showing pathogenic differences among Erwinia isolates.

Graham and Dowson (1960) and Graham (1972) reported that temperature and site of inoculation were major factors in establishing infection. Inoculum had to be introduced into the vascular system of the plant to obtain infection. However, the temperature at which inoculated plants were incubated was shown to be important. Graham and Dowson (1960) tested a number of isolates obtained from different hosts in different countries. They reported that all isolates produced blackleg symptoms when inoculated plants were held at 24.5°C or above. However, only a few of the isolates could produce blackleg at 19°C and below. Thus, Graham and Dowson (1960) grouped isolates into two categories: 1) isolates producing blackleg at high temperatures only and 2) isolates producing blackleg at high and low temperatures. The first group consisted mainly of isolates obtained from plants originating in tropical or subtropical countries or from plants cultivated in hot houses. The second group was almost exclusively derived from plants in temperate regions. Most of the isolates in the latter group were regarded as E. carotovora var. atroseptica.

All of these studies showed that temperature and site of inoculation were important factors in influencing disease development. However, more information is needed to establish a significant relationship between physiological and pathological characteristics of E. carotovora (Graham and Dowson, 1960; Graham, 1964).

Effect of temperature on growth:

Minimum and optimum temperatures for growth are 3°C and 27°C, respectively, for both varieties of E. carotovora (Bergey, 1974). Maximum temperatures for growth are reported to be 37-41°C for E.

carotovora var. carotovora and 35°C for E. carotovora var. atroseptica (Bergey, 1974). However, detailed information on growth rates of E. carotovora at various temperatures is lacking.

Erinle (1975) has studied the effects of temperatures on growth, in vivo, of E. carotovora. He reported differences between varieties of E. carotovora when inoculated plants were incubated at high and low temperatures. At high temperatures all isolates grew logarithmically for varying lengths of time. Then, E. carotovora var. atroseptica isolates continued to grow and incite blackleg whereas E. carotovora var. carotovora isolates remained stationary or decreased. At low temperatures the growth of both varieties of E. carotovora was retarded, especially E. carotovora var. carotovora.

Etiology:

Many researchers (Graham and Dowson, 1960; Lazur and Bucur, 1964; Logan, 1968; Graham and Hardie, 1971; Perombelon, 1971) have reported that E. carotovora var. atroseptica is the causal agent for blackleg. In contrast, Stanghellini and Meneley (1975) identified the primary causal bacterium of blackleg in Arizona as E. carotovora var. carotovora. Recently, Molina and Harrison (1977) reported that although both varieties of E. carotovora were isolated from blackleg plants in Colorado, E. carotovora var. atroseptica was the predominant causal agent. E. carotovora var. carotovora was isolated mainly from blackleg plants grown in warmer areas of Colorado.

It has been shown repeatedly (Bonde, 1950; Graham, 1962; Harper, Boyd and Graham, 1963; Graham and Harper, 1967) that blackleg plants are the major source of contamination of new tubers. Thus, infected

tubers should be those produced by blackleg plants and their immediate neighbours. The results of Pérombelon (1972a) indicated that tuber contamination within Scottish stocks was extensive and that the majority of harvested tubers were contaminated by E. carotovora regardless of blackleg infections in the field. For these reasons, Pérombelon (1972a) concluded that contaminated seed tubers were the major source of primary inoculum for blackleg disease.

Pérombelon (1974) conducted several experiments using plants produced with and without mother tubers. He observed that progeny tubers were never contaminated with E. carotovora in the absence of mother tubers and while the mother tubers remained sound. Contamination of daughter tubers did occur after the mother tubers began to rot.

Field experiments monitoring the levels of daughter tuber contamination during the growing season were conducted by Pérombelon (1969b). Extensive contamination of daughter tubers occurred following the generalized rotting and breakdown of the mother tubers. From that point, contamination levels fluctuated markedly from week to week. Later in the season (October/November) contamination fell to a low level although it was never absent.

The results of Pérombelon (1969b and 1974) supported his conclusion that contaminated seed tubers were the major source of E. carotovora for the growing crop. However, it became apparent that other factors affected manifestation of the disease in the field (Pérombelon, 1972a). Many researchers (Gratz, 1930; Bonde, 1950; Conroy, 1952; Graham, 1962; Logan, 1968) have reported that contaminated seed tubers may or may not produce blackleg plants in the field. Bonde (1950)

observed that blackleg infections in growing crops varied tremendously from year to year.

Erinle (1974) reported that a minimum threshold population of 10^8 cells per gram of stem was a prerequisite for disease expression. Various factors such as inoculum concentration, environmental conditons, and host susceptibility could affect the build-up of bacterial populations to the minimum threshold level.

Maas Geesteranus and Vruggink (1976) studied the relationship of seed tuber contamination and disease expression in the field. These researchers concluded that the appearance of blackleg symptoms was related to the depth of bacterial penetration within tubers. More specifically, tubers formed in wet soil allowed deep penetration of the bacteria due to the open lenticels and a continuous water film around the tubers. After sprouting, the bacteria moved quickly into the stem causing blackleg symptoms to appear early in the growing season. Conversely, lenticels and wounds tended to be practically closed and the intercellular spaces filled with air under drier conditions. This slowed down bacterial penetration of the tubers resulting in symptom expression later in the season.

Detection of Erwinia carotovora in tubers

Perombelon (1972b) developed a reliable method for detecting E. carotovora in tubers. Tubers were incubated under conditions of low oxygen and high humidity to induce rotting. The growth and multiplication of strict and facultative, pectolytic anaerobes is favoured, especially Clostridium spp. Bacillus spp. and E. carotovora.

De Boer and Kelman (1975) evaluated various procedures for detecting

pectolytic E. carotovora in potato tubers. They developed a simple method of wrapping tubers in moist paper towelling followed by two layers of polyvinylidene film. This method is based on the same principles as that of Pérombelon (1972 b) and is reported to be effective in detecting low numbers of E. carotovora in tubers (De Boer and Kelman, 1975).

Recently, Burr and Schroth, (1977) reported on a direct lenticel isolation technique. Ten lenticels plus the stem end of each tuber were stabbed with a sterile wooden toothpick and the toothpick streaked across a selective medium between stabbings. This method enabled Burr and Schroth to test tubers for E. carotovora within a 48 hour period.

Pérombelon (1972 b), De Boer and Kelman (1975), and Burr and Schroth (1977) have reported that tuber contamination by E. carotovora is widespread. These researchers estimated percent contamination of E. carotovora var. carotovora and E. carotovora var. atroseptica from a random sample of pectolytic isolates obtained from rotting tubers. Pérombelon (1972 b) reported that approximately 80% of the Erwinia isolates obtained from Scottish stocks were identified as E. carotovora var. atroseptica. The remaining 20% were E. carotovora var. carotovora. De Boer and Kelman (1975) identified a random sample of E. carotovora isolates of which forty-five percent were E. carotovora var. atroseptica and 55% were E. carotovora var. carotovora. Burr and Schroth (1977) reported that both varieties of Erwinia carotovora were isolated from potato seedlots and that a large proportion of the selected isolates could not be identified as either E. carotovora var. carotovora or E. carotovora var. atroseptica.

The lenticels were found to be the most important site of contamination either in or on the potato tuber (Pérombelon, 1973b). E. carotovora can survive in the lenticels and on the tuber surface throughout storage. However, various researchers (Pérombelon and Lowe, 1975; De Boer and Kelman, 1975) reported that the number of viable bacteria and the ability to detect them decreases with time in storage.

Pérombelon and Lowe (1972) demonstrated the importance of surface inoculum. They correlated bacterial numbers on the tuber surface with blackleg incidence in the crop the following year. E. carotovora was also detected in the vascular ring of tubers harvested from blackleg plants, but this contamination was thought to be of minor significance (Pérombelon and Lowe, 1972).

Soil contamination:

There is still controversy on whether or not the soft rotting Erwinias are natural soil inhabitants (Buddenhagen, 1965). Buddenhagen has assessed the role of the soil in the biology of many bacterial plant pathogens. He has characterized the soft rot Erwinias as pathogens that build-up populations within susceptible hosts but whose populations gradually decline in the soil. The E. carotovora varieties can not be considered true soil bacteria since their long term presence in soils is host dependent. However, these pathogens can persist as soil saprophytes in association with the rhizosphere of weed hosts (Copeman and Schneider, 1975).

Early studies by Leach (1930), Patel (1929) and Bonde (1950) indicated that soft rotting Erwinia survived in the soil for varying lengths of time. Other researchers (Graham, 1958; Graham, 1962;

Vorckovich, 1960; Lazur and Bucur, 1964; Logan, 1968; Pérombelon, 1970; Graham and Hardie, 1971; Pérombelon and Lowe, 1972; Cuppels and Kelman, 1974; De Boer et al., 1974) could not isolate E. carotovora from soil. They concluded that E. carotovora was not free-living in the soil. The evidence supporting these conclusions has been summarized by Graham and Hardie, 1971 as follows: 1) E. carotovora could not be isolated from soils in spring just prior to planting potatoes, 2) populations of E. carotovora declined to undectable levels in a matter of months in both artificially inoculated and naturally contaminated soils, 3) E. carotovora was never isolated from ponds and streams, 4) E. carotovora rarely infected other host plants at least in Scotland.

Selective media have been used in all of the above mentioned studies for the recovery of E. carotovora from artifically inoculated and field soil. Cuppels and Kelman (1974) and Burr and Schroth (1977) have indicated that this technique is insensitive for detecting low populations of Erwinia spp. in soil.

Meneley and Stanghellini (1976) reported on a soil enrichment technique which enabled them to isolate E. carotovora from field soil. The enrichment medium supported the rapid growth of E. carotovora and eliminated the competitive growth of aerobic, pectolytic organisms. Low populations of bacteria in the range of two to seven cells per gram of soil were detected. The enrichment technique did not allow direct quantitative comparisons but did increase the sensitivity of isolating E. carotovora from soil 100 - 1,000 fold (Stanghellini and Meneley, 1975) compared with isolation procedures with selective media (Logan, 1968; Togashi, 1972; Cuppels and Kelman, 1974).

Burr and Schroth (1977) reported similar results. E. carotovora was isolated from soil with an enrichment technique both aerobically and anaerobically with the latter improving selectivity. E. carotovora was isolated mainly from field soils in which plant debris was visible and even from some soils without the aid of enrichment. Survival of E. carotovora var. atroseptica in soil and the rhizosphere of weed species has also been implicated as an important potential reservoir of inoculum under British Columbia conditions (Copeman and Schneider, 1975).

Mew et al. (1976) have concluded that E. carotovora var. carotovora is widely distributed in the soil in Japan. They have shown that Erwinia spp. are present but undetectable in the absence of the host crop and that populations increased to detectable levels during the growth of the susceptible host, Chinese cabbage.

Meneley and Stanghellini (1976) and Burr and Schroth (1977) have identified a random sampling of E. carotovora isolates obtained from field and rhizosphere soil using the anaerobic enrichment technique. The majority of the isolates were E. carotovora var. carotovora. Only a small percentage of the pectolytic isolates were E. carotovora var. atroseptica.

Logan (1968) and Meneley and Stanghellini (1976) reported that survival of E. carotovora increased when plant material was present in the soil. This observation is supported by Ficke et al. (1968) who found that long frosty periods, snow cover, and straw and plant residues prolonged the life of E. carotovora var. atroseptica in soils.

Soil temperatures greater than 20°C are reported to decrease survival of E. carotovora (Pérombelon, 1974). However, Meneley and Stanghellini (1976) isolated E. carotovora from certain soils that

had undergone a summerfallow. The initial population of these bacteria had to survive in soils where summer temperatures ranged from 18-59.5°C (Avg. 25°C) at the 12.5 cm depth.

Insect transmission:

Leach (1930) reported on certain insects commonly associated with blackleg and seed piece decay including the common housefly (Musca domestica), false cranefly (Trichocera sp.), fungus gnat (Sciara tribentala) and two species of fruit flies (Drosophila funebris and Drosophila bruschii). Most of these insects were considered to be merely scavengers feeding on the decayed vegetable matter and did not appear to be factors in the dissemination of these diseases.

Many insects of the genus Drosophila found near rubbish heaps, potato rejects and garden refuse have been reported by many researchers (Leach, 1930; Stapp, 1961; Pepper, 1967; Mazzucchi and Dalli, 1973) to transmit the organisms causing blackleg and soft rot. The potato blackleg organism, E. carotovora var. atroseptica can live in all stages of the seed-corn maggot (Hylemya platura) and may persist in this insect, even though it can also persist in tubers and soil (Schuster and Coyne, 1974). The seed-corn maggot increases disease incidence by transporting the pathogen to favourable infection sites. Leach (1940) demonstrated that E. carotovora var. atroseptica was present in the intestinal tracts of both adult flies and larvae of the seed-corn maggot. The blackleg organism has also been isolated from insects, especially fruit flies, in the field even in the absence of potato plants (Graham and Hardie, 1971).

Control:

Since potatoes are vegetatively propagated certain problems of disease control arise which are either non-existent or of lesser importance in plants that are reproduced from seed. Pathogens already existing in infected propagative material can continue to develop in the progeny. As well, cut surfaces of tubers and stem cuttings provide open courts for infection.

Use of tested cuttings for production of healthy plants is not new. The technique for control of blackleg by using stem cuttings was developed at East Craigs, Scotland in 1967 to minimize blackleg incidence in propagative material and to provide relatively disease-free (certified) planting stock to growers. This program is now used in the large scale production of disease-free seed throughout Europe and North America. Blackleg-free stocks are now considered to be a possible solution for controlling blackleg (Graham and Harper, 1967; Perombelon et al., 1976).

Graham and Hardie (1971) reported on the results of testing cuttings from 1967-1971 at East Craigs. During the five year period 9,300 cuttings were tested and none was infected. The bacteria were unable to multiply in the mother tuber and spread into the stem before the cuttings were taken. However, the blackleg organism was recovered from cuttings of ordinary commercial stocks examined in July. Consequently, cuttings are not used for routine propagation after mid-June and never from field plots (Graham and Hardie, 1971).

Levels of blackleg within commercial virus-tested stem cutting crops have been estimated from field inspections. Graham and Hardie

(1971) reported that sixty four of two hundred and seventy acres (24%) were rejected for blackleg in 1971. Infections ranged from .001-.57% within these rejected stocks.

Re-contamination of virus tested stem cutting stocks has been reported by other researchers (Pérombelon, 1974; Pérombelon and Lowe, 1976; Molina and Harrison, 1977). E. carotovora var. carotovora has been detected most frequently within these stocks (Pérombelon, 1974; Pérombelon and Lowe, 1976; Molina and Harrison, 1977). Surveys conducted by Pérombelon and Lowe (1976) showed that approximately one-quarter of the stocks were contaminated by E. carotovora var. carotovora after only one growing season. Contamination levels increased rapidly between and within stocks and almost all stocks were heavily infected by the fifth year (Pérombelon and Lowe, 1976).

At present, the only means of controlling recontamination is through strict hygienic procedures (Graham and Harper, 1967; Pérombelon, 1974). Several compounds have been used as surface disinfectants on cutting knives, storages and equipment to control blackleg. Quaternary ammonium compounds, hypochlorite and chlorinated phenols were tested as disinfectants. These chemicals were readily inactivated on contact with either soil or plant debris and proved ineffective against E. carotovora (Graham and Hardie, 1971). Five percent formaldehyde solution containing a wetting agent was reported to be an effective disinfectant by these same authors. Letal (1977) observed that mercuric chloride (0.1%) and formaldehyde (2% and 5%) were the most effective against E. carotovora var. atroseptica.

Attempts to control blackleg by disinfecting tuber surfaces

have proven ineffective. Graham and Volcani (1961) and Graham and Harper (1967) reported that sodium hypochlorite and mercurial compounds either with or without antibiotics failed to prevent blackleg. Spraying or dipping the tubers does not allow chemical penetration of the lenticels. This was considered to be the main reason for the ineffectiveness of chemical treatments.

Spraying the growing crop with antibacterial preparations failed to reduce blackleg infections (Graham and Harper, 1967). Attempts to breed for resistance against soft rot and blackleg have not been successful (Graham and Harper, 1967).

At present, control measures must be based on agronomic practices and should aim at reducing the spread of the organism in the growing crops. This includes planting healthy, sound, certified disease-free tubers, roguing out infected plants and preventing tuber contamination at lifting, grading and during storage (Graham and Harper, 1967). Chemical control through the use of an active, systemic bactericide is seen as a possible solution for the elimination of blackleg in future years.

PART ONE

Incidence of Erwinia carotovora within Manitoba potatoes

INTRODUCTION

Numerous studies (Burkholder and Smith, 1949; Bonde, 1950; Graham and Dowson, 1960; Graham, 1963; Graham and Harper, 1967; Logan, 1968; Graham and Hardie, 1971; Pérombelon, 1971; Vruggink and Maas Geesteranus, 1975) showed that blackleg and tuber soft rot were due to infection by E. carotovora var. atroseptica. These same authors claimed that the related organism, E. carotovora var. carotovora caused only tuber soft rot and did not contribute to blackleg infections in the field. Recent studies by Stanghellini and Meneley (1975) and Molina and Harrison (1977) indicated that E. carotovora var. carotovora appears to cause both tuber soft rot and blackleg in the field.

Therefore, the present study was conducted to investigate the presence of both varieties of E. carotovora in Manitoba potatoes by monitoring the nuclear stock of the stem cutting program and the commercial stock.

MATERIALS AND METHODS

Origin of the Tested Cuttings:

Mother tubers representing the nuclear stock of Manitoba's elite stem cutting program were tested for soft rot Erwinia spp. contamination in the spring of 1976, 1977 and 1978 using a method based on the one developed at East Craigs Laboratory, Scotland.

Procedure for testing nuclear stock cuttings:

One two-inch cutting taken from the base of each nuclear stock plant was macerated in a Waring blender (capacity 50 ml) with 15 ml of sterile, distilled water. The macerates were allowed to settle for one hour and then 0.1 ml of suspension streaked onto crystal violet pectate (CVP) medium. The plates were incubated for 48 hours at 24°C and then checked for the appearance of deep pits in the medium.

All pitted colonies were streaked on nutrient agar plates to ensure that each culture was pure. Pure cultures of each isolate were maintained on nutrient agar slants and identified as either Pseudomonas spp. or soft rot Erwinia spp. on the basis of the results of several biochemical tests.

Isolates which were gram-negative, oxidase-negative, fermented glucose within 48-72 hours and did not fluoresce on King's Medium B were classified as soft rot Erwinia spp. Isolates were identified as Pseudomonas spp. if they were gram-negative, oxidase-negative, unable

to ferment glucose within 48-72 hours and able to fluoresce on King's Medium B.

Five additional biochemical tests listed in Table 2 were conducted with all soft rot Erwinia spp. isolates to identify them as either E. carotovora var. carotovora or E. carotovora var. atroseptica.

Origin of the Commercial Stock Tubers:

Netted Gem and Norland potatoes selected at random from 28 growers were tested for soft rot Erwinia spp. contamination during the fall and winter of 1977.

Procedure for Testing Commercial Stock:

The procedure for testing tubers was modified from Perombelon, 1972b. Three tubers from each five-pound sample lot were placed in individual polyethylene bags containing five ml of sterile, distilled water. These bags were left open and three of them placed in a larger polyethylene bag containing 50 ml of sterile, distilled water. The larger bags were tied securely and incubated on the laboratory bench at room temperature (approximately 25°C). Tubers were periodically checked for early signs of break down and any such tubers removed from the bags.

Tissue from the edge of the rotted area was suspended in 15 ml of sterile, distilled water contained in 20 ml culture tubes. The tubes were incubated for one hour at room temperature to allow the suspended particles to settle. Aliquots of suspensions (0.1 ml) were streaked onto CVP medium and the plates incubated at 24°C for 48-72 hours. Plates were checked for any evidence of pitting and two pitted

TABLE 2. Varietal differentiation of *Erwinia carotovora*.

TEST	VARIETY CAROTOVORA	VARIETY ATROSEPTICA
Growth at 37°C	+	-
Production of reducing compounds from sucrose	-	+
Acid from α-methyl glucoside	-	+
Reaction with atro-septica antiserum	-	+
Pathogenicity	-	+

colonies per plate were streaked individually on nutrient agar plates. Identification procedures described previously were performed with all purified isolates.

Procedure for isolating from Blackleg plants:

During the summer of 1977 a total of 21 blackleg plants were collected from various potato fields. Sections of the lower stem close to the edge of the rot were surface sterilized in 2% sodium hypochlorite, rinsed in sterile water and macerated in 10 ml of sterile, distilled water in a Waring blender. The macerates were poured into sterile tubes and suspended particles allowed to settle. Aliquots of these suspensions (0.1 ml) were streaked onto CVP medium and the plates incubated at 25°C for 48-72 hours. From one to four pitted colonies per plate were purified on nutrient agar plates and maintained on nutrient agar slants. Identification procedures described previously were performed with all purified isolates.

RESULTS

Nuclear Stock Testing:

The results of testing nuclear stock plants in the stem cutting program during 1976, 1977 and 1978 are listed in Table 3. Some mother tubers were contaminated with E. carotovora var. atroseptica and E. carotovora var. carotovora in the first two years of testing. In 1978 all mother tubers were free from Erwinia spp. contamination.

The 1976 and 1977 test results have been tabulated by potato variety in Tables 4 and 5, respectively. In 1976 nine varieties were tested and only one of these, Chieftain, was contaminated with E. carotovora var. atroeptica. E. carotovora var. carotovora was detected in six varieties including Netted Gem, Norland, Norchip, Targee, Red Pontiac and Chieftain. In 1977 when only four varieties were tested, E. carotovora var. atroseptica infections were limited to the Norland variety and E. carotovora var. carotovora to the Netted Gem variety.

Commercial Stock Testing:

The commercial stock testing results are recorded in Table 6. One hundred and sixty-one of the 271 sampled tubers (59%) rotted due to contamination by soft rot Erwinia spp. Two hundred and eight isolates were selected at random from these rotting tubers and identified to variety. Eighty-nine percent of these isolates were identified as E. carotovora var. carotovora and 5% as E. carotovora var. atroseptica.

TABLE 3. Results of testing Manitoba's Nuclear Stock.

YEAR	TUBER CONTAMINATION					
	E. carotovora var. *atroseptica*		*E. carotovora* var. *carotovora*		*Pseudomonas* spp.	
	No.	%	No.	%	No.	%
1976	1/149	1	23/149	15	8/149	5
1977	5/49	10	1/49	2	17/49	35
1978	0/46	0	0/46	0	12/46	26

TABLE 4. Results of testing Manitoba's Nuclear Stock -- 1976.

VARIETY	TUBER CONTAMINATION					
	E. carotovora var. *atroseptica*		*E. carotovora* var. *carotovora*		*Pseudomonas* spp.	
	No.	%	No.	%	No.	%
Netted Gem	0/34	0	11/34	32	1/34	3
Norland	0/26	0	3/26	12	1/26	4
Norchip	0/15	0	3/15	20	1/15	7
Kennebec	0/15	0	0/15	0	1/15	7
Targee	0/12	0	2/12	17	0/12	0
Red Pontiac	0/10	0	3/10	30	1/10	10
Chieftain	1/11	9	1/11	9	2/11	18
Bison	0/18	0	0/18	0	0/18	0
FS 6339	0/8	0	0/8	0	1/8	13

TABLE 5. Results of testing Manitoba's Nuclear Stock -- 1977.

VARIETY	TUBER CONTAMINATION					
	E. carotovora var. *atroseptica*		*E. carotovora* var. *carotovora*		*Pseudomonas* spp.	
	No.	%	No.	%	No.	%
Netted Gem	0/24	0	1/24	4	16/24	67
Norland	5/9	56	0/9	0	1/9	11
Norchip	0/8	0	0/8	0	0/8	0
Kennebec	0/8	0	0/8	0	0/8	0

TABLE 6. Commercial stock testing results-1977.

VARIETY	TUBERS ROTTING		ORGANISMS RECOVERED					
			Erwinia carotovora					
			variety atroseptica		variety carotovora		Atypical	
	No.	%	No.	%	No.	%	No.	%
NETTED GEM	7/24	29	1/8	13	7/8	88	0/8	0
NORLAND	154/247	62	10/200	5	179/200	90	11/200	6
TOTAL	161/271	59	11/208	5	186/208	89	11/208	5

An additional 5% could not be identified as either variety and were listed as atypical.

Isolations from Blackleg Plants:

A total of forty isolates were obtained from twenty-one blackleg plants. The majority (98%) of these isolates were identified as E. carotovora var. atroseptica. Only one of the forty isolates (2%) was identified as E. carotovora var. carotovora.

DISCUSSION

The nuclear stock within a potato stem cutting program must be continually monitored for the presence of E. carotovora (Graham and Harper, 1967; Graham and Hardie, 1971; Pérombelon and Graham, 1973; Hardie, 1976; Pérombelon, Lowe and Ballantine, 1976). The testing of Manitoba's nuclear stock plants showed infections by both E. carotovora var. carotovora and E. carotovora var. atroseptica during 1976 and 1977. Until recently, only E. carotovora var. atroseptica was reported to cause blackleg infections (Burkholder and Smith, 1949; Bonde, 1950; Graham and Dowson, 1960; Graham, 1963; Graham and Harper, 1967; Logan, 1968; Graham and Hardie, 1971; Pérombelon, 1971; Vruggink and Maas Geesteranus, 1975). Contaminated mother plants and all cuttings derived from them were discarded if this organism was detected. However, recent reports by Stanghellini and Meneley (1975) and Molina and Harrison (1977) have shown that E. carotovora var. carotovora can contribute to blackleg infections in the field. These findings are important since the 1976 and 1977 testing of Manitoba's nuclear stock indicated contamination by E. carotovora var. carotovora.

The predominant organism isolated from blackleg plants in the field in Manitoba was E. carotovora var. atroseptica. These findings are in agreement with the reports of many researchers (Lazur and Bucur, 1964; Pérombelon, 1972a; Vruggink and Maas Geesteranus, 1975; Molina and Harrison, 1977) who have concluded that E. carotovora var. atrosep-

tica was mainly responsible for blackleg in the field.

Molina and Harrison (1977) have reported that the occurrence of stem infections caused by E. carotovora var. carotovora appeared to be greatly influenced by geographic location and that this organism was isolated almost exclusively from infected plants grown in warmer areas of Colorado. This relationship of high temperatures and frequency of E. carotovora var. carotovora infections is supported by the results of Stanghellini and Meneley (1975). These researchers reported that E. carotovora var. carotovora was the major causal agent of blackleg in Arizona. The infrequent recovery of E. carotovora var. carotovora from blackleg plants in Manitoba indicates that cooler temperatures prevailing in this region reduce infection by this organism and increase the frequency of infection by E. carotovora var. atroseptica.

Some of the isolates recovered from the stored tubers could not be identified as either E. carotovora var. carotovora or E. carotovora var. atroseptica. This difficulty has been reported by various researchers (Stanghellini and Meneley, 1975; Meneley and Stanghellini, 1976; Burr and Schroth, 1977) and questions the reliability and validity of the tests currently used in varietal differentiation. However, the five biochemical tests previously described are reported to be the most reliable criteria for differentiation between E. carotovora var. carotovora and E. carotovora var. atroseptica. Expanded surveying of blackleg plants and potato stocks may reveal an even greater range of variability among soft rot Erwinia spp. isolates.

PART TWO

The effect of low temperature on soil survival of *Erwinia carotovora*

INTRODUCTION

Various researchers have attempted to assess the role of soil survival in the etiology of the blackleg-soft rot disease complex (Leach, 1931; Bonde, 1950; Graham, 1958; Logan 1969; Ficke *et al*. 1973; De Boer *et al*. 1974; Pérombelon, 1974; Meneley and Stanghellini, 1976; Burr and Schroth, 1977). Many have utilized selective media and reported that *Erwinia* spp. survive for only a short time in soils (Logan, 1969; De Boer *et al*., 1974; Pérombelon, 1974). Furthermore, it was presumed that low soil temperatures during winter months adversely affected bacterial populations resulting in their rapid decline in field soil in temperate climates (Logan, 1969; Pérombelon, 1974). More sensitive techniques involving enrichment culture have enabled detection of low numbers of *Erwinia* spp. in field soil in recent years (Meneley and Stanghellini, 1976; Burr and Schroth, 1977).

In the present study a series of five experiments was conducted to determine whether *E. carotovora* var. *carotovora* and *E. carotovora* var. *atroseptica* could overwinter in the field soil under Manitoba climatic conditions.

MATERIALS AND METHODS

Selection of isolates:

Two isolates of E. carotovora var. carotovora recovered from potato tubers and two of E. carotovora var. atroseptica isolated from blackleg plants were selected at random to study survival of soft rot Erwinia spp. in soil and tubers under controlled and natural environments.

Preparation of inoculum:

Three methods were used in the preparation of inoculum for these experiments.

METHOD A. One isolate of E. carotovora var. carotovora and one isolate of E. carotovora var. atroseptica were streaked individually on four nutrient agar plates and the plates incubated at 25°C for 48 hours. Plates were flooded with 10 ml of sterile, distilled water per plate providing a total of 40 ml per isolate. Each suspension was added to an Erlenmeyer flask containing 500 grams of peeled, cubed, steam-sterilized potatoes. Both Erlenmeyer flasks were fitted with cotton plugs and incubated at 25°C for five days.

METHOD B. Two isolates of E. carotovora var. carotovora and two isolates of E. carotovora var. atroseptica were streaked individually on two nutrient agar slants and the tubes incubated at 25°C for 24 hours. Tubes were flooded with five ml of sterile, distilled water per tube providing

a total of 10 ml per isolate. Each suspension was added to an Erlenmeyer flask containing 500 ml of potato broth. The four flasks were placed on a shaker tray at room temperature for 48 hours.

METHOD C. One isolate of E. carotovora var. carotovora and one isolate of E. carotovora var. atroseptica were streaked individually on two nutrient agar plates and incubated at 25°C for 48 hours. Plates were flooded with 10 ml of sterile, distilled water per plate providing a total of 20 ml per isolate. The final volume of each suspension was adjusted to 300 ml with sterile, distilled water.

Soil inoculation:

Three methods of soil inoculation were compared in this study.

METHOD 1: Rotted potato inocula prepared according to method A were mixed with unsterile, sandy-loam soil in the proportions 1 part rotted potato: 9 parts soil and blended to form a homogeneous mixture. Each soil-potato rot mixture was divided into three equal portions and each portion packed in a six-inch plastic pot. Each pot was incubated at one of three temperatures, +20°C, +5°C and -10°C. Pots were held at decreasing temperatures for several hours permitting soil temperatures to drop slowly. This procedure was repeated two more times yielding three replications of each bacterial isolate at each of the three temperatures.

METHOD 2. This method was the same as that described above except that soil samples of twenty-five grams were placed in small, sterile, polyethylene (Whirl-pak) bags. Thirty bags per isolate were filled to provide three replications of ten sampling dates. All bags were placed in metal containers and the containers buried underground throughout the fall and

winter. This procedure was repeated and two trials were conducted.

METHOD 3: Twenty-gram portions of soil were weighed and placed in sterile, polyethylene (Whirl-pak) bags. Aliquots of potato broth inocula (1.0 ml) prepared as described previously in Method B were added to the soil in the bags and the bags well shaken. A total of 40 bags per isolate were inoculated yielding four replications of 10 sampling dates. The bags were placed in metal containers and the containers buried underground during the fall and winter.

Tuber inoculation:

Tubers were stabbed several times with a sterile, wooden toothpick and inoculated by dipping for one minute in bacterial suspensions prepared as described previously in Method C. Fifty tubers were inoculated with each bacterial variety. Tubers were placed in polyethylene bags and incubated at 25°C for 72-96 hours. Then, bags were opened and filled with sandy, loam soil. Forty tubers (twenty per isolate) were buried at Site I on September 22, 1977 and sixty tubers (thirty per isolate) were buried on October 3, 1977 at Site II. Tubers were left in the ground during the fall and winter.

Bacterial recovery:

Four procedures of bacterial recovery were employed in this study corresponding to the three methods of soil inoculation and single method of tuber inoculation.

METHOD 1: Soil samples were taken at week zero, one, two, three, six, nine and twelve by removing 25 g of soil with a sterile cork borer. Twenty grams of soil per sample were weighed, suspended in 200 ml of

sterile, distilled water and shaken for one hour. Soil suspensions were serially diluted and 0.1 ml aliquots of each dilution (1×10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) were plated on CVP medium. The plates were incubated for 48-72 hours at 25°C, after which pitted colonies could be easily counted.

METHOD 2. Six bags (three bags per isolate) were collected in each trial on designated dates throughout the fall and winter. In the first trial soil samples were taken on Sept. 12, Sept. 26, Oct. 10, Oct. 24, Nov. 7, Jan. 16, April 10, April 24, May 8 and May 22. Samples were taken on alternate weeks in the second trial and these dates were Sept. 19, Oct. 3, Oct. 17, Oct. 31, Nov. 14, Jan. 16, April 17, May 1, May 15 and May 29. Soil samples (20 g) were removed from each bag and suspended in 200 ml of sterile, distilled water. Suspensions were treated as described above.

An enrichment culture technique was employed for recovering E. carotovora var. atroseptica from soil sampled on April 24, May 8 and May 22 in the first trial and from soil samples on May 15 and May 29 in the second trial. This procedure consisted of removing the soil samples from the bags and placing the soil in sterile, petri dishes. Ten ml of PT broth (Appendix I) were added and the dishes placed in anaerobic jars (BBL Gas Pak^{R} System) at room temperature (25°C). Soil samples were transferred to sterile, culture bottles after 48 hours and 190 ml of sterile, distilled water were added. Culture bottles were placed on a shaker tray at room temperature for one hour. Soil suspensions were plated out following the procedure previously described.

METHOD 3. Sixteen bags (four bags per isolate) were sampled on the

following dates: Oct. 20, Nov. 3, Nov. 17, Dec. 1, Jan. 12, April 13, April 27, May 11, May 25 and June 8. The contents of each bag were suspended in 200 ml of sterile, distilled water in culture bottles. Soil suspensions were plated out following the method previously described. The enrichment culture technique was employed for recovering soft rot Erwinia spp. from soil sampled on April 13, April 27, May 11, May 25 and June 8.

METHOD 4: Ten bags (five bags per isolate) were sampled on April 17, May 3, May 30 and June 28 from site I and May 2, May 10, May 17, May 23, June 21, and June 26 from site II.

Soil and tubers were removed from the bags. Twenty grams of soil were weighed and placed in sterile, petri dishes. Ten ml of PT broth were added. Rotted tubers were opened aseptically and 10 grams of inner, rotted tissue placed in sterile, petri dishes containing 10 ml of PT broth. Plates were incubated in anaerobic jars at room temperature for 48 hours. Soil samples were transferred to culture bottles and 190 ml of sterile, distilled water added. Similarly, tissue samples were placed in culture bottles containing 90 ml of sterile, distilled water. Soil and tissue suspensions were plated according to methods previously described.

RESULTS

Mean numbers of E. carotovora var. carotovora and E. carotovora var. atroseptica recovered from soil incubated at +20°C, +5°C and -10°C are illustrated in Figure 1 and 2, respectively. There were no significant differences between bacterial varieties in the length of time cells remained viable in soil. However, survival of soft rot Erwinia spp. in soil proved to be significantly different with temperature (Appendix IIb). Soft rot Erwinia spp. declined very rapidly in soil incubated at +20°C and after six weeks, cells were no longer detected. Both organisms showed a greater ability to survive in soil incubated at the lower temperatures. Cells were still recovered from soil after a 12 week incubation period at +5°C and -10°C. Survival of soft rot Erwinia spp. was greatest at +5°C and resulted in the recovery of large numbers of cells from soil incubated at this temperature at the end of 12 weeks.

Figure 3 and 4 illustrate the mean numbers of E. carotovora var. carotovora and E. carotovora var. atroseptica recovered from over-wintered soil in two trials (September 12 to May 22 and September 19 to May 29), respectively, The results were similar in both trials and indicated that large populations of E. carotovora var. carotovora remained viable throughout the winter. In the first and second trial, 1.5×10^7 and 1.7×10^5 cells per gram of soil, respectively were recovered at the end of 36 weeks. In contrast, E. carotovora var. atroseptica declined more rapidly and was not recovered from soil with the selective medium after 30 weeks. For this reason, a more sensitive technique of enrichment culture prior to plating was used for the last

FIGURE 1. Mean numbers of Erwinia carotovora variety carotovora recovered from soil incubated at various temperatures (+20°C, +5°C and -10°C).

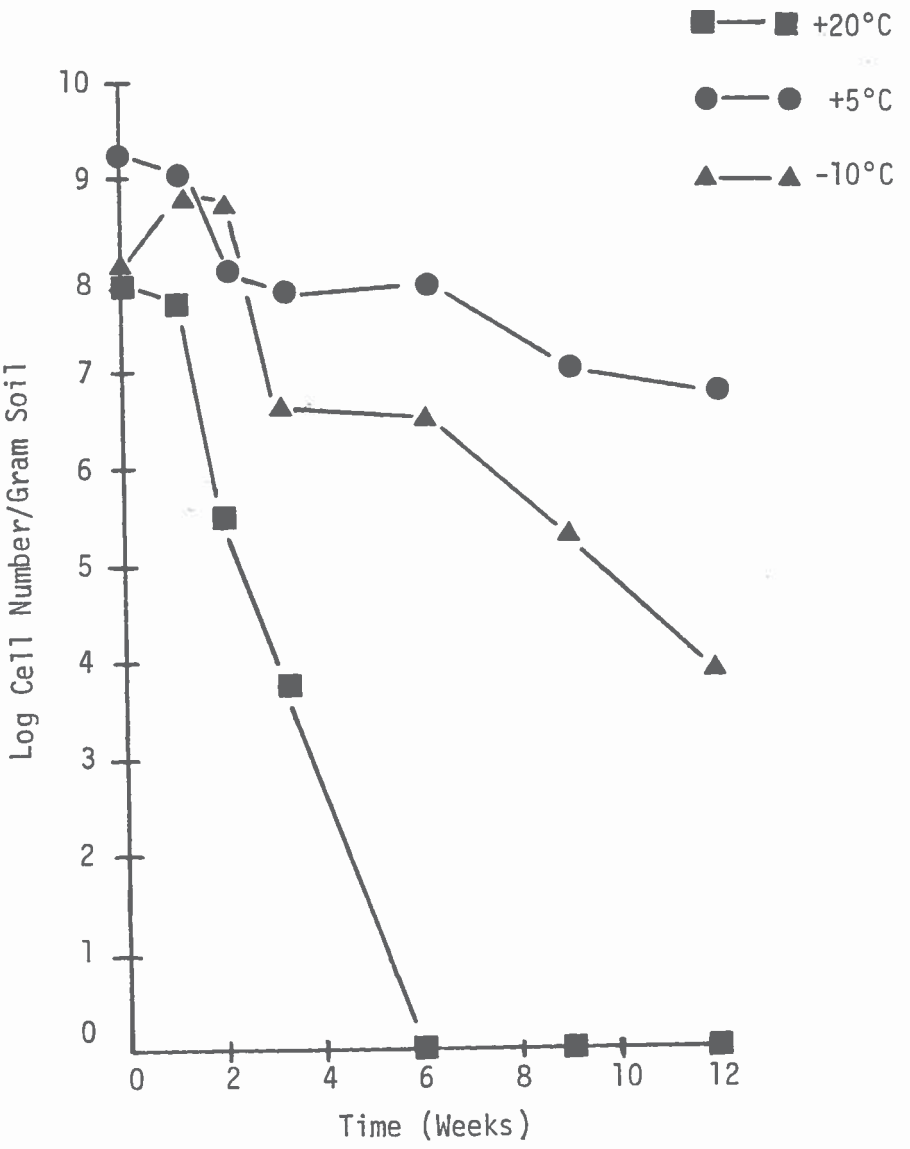


FIGURE 2. Mean numbers of Erwinia carotovora variety atroseptica recovered from soil incubated at various temperatures (+20°C, +5°C and -10°C).

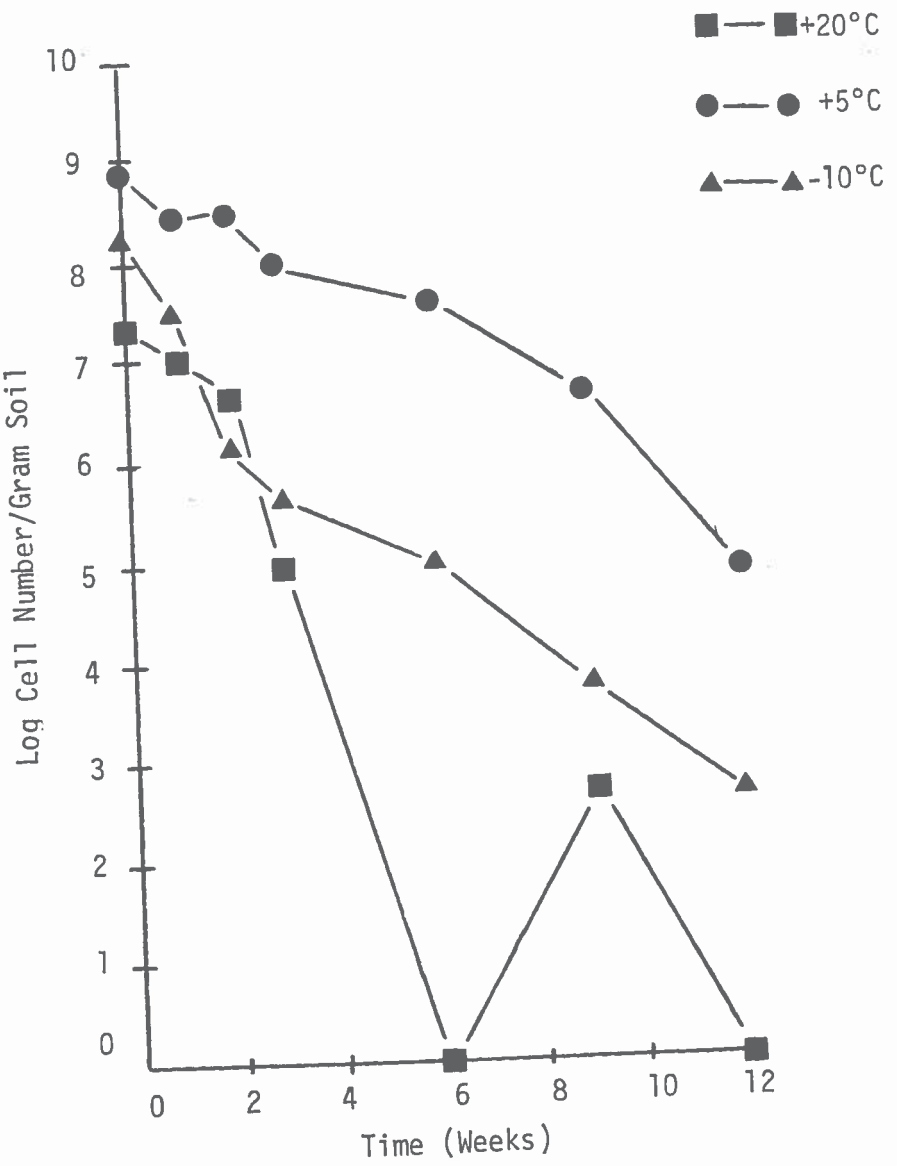


FIGURE 3. Mean numbers of Erwinia carotovora recovered from overwintered soil (September 12 to May 22).

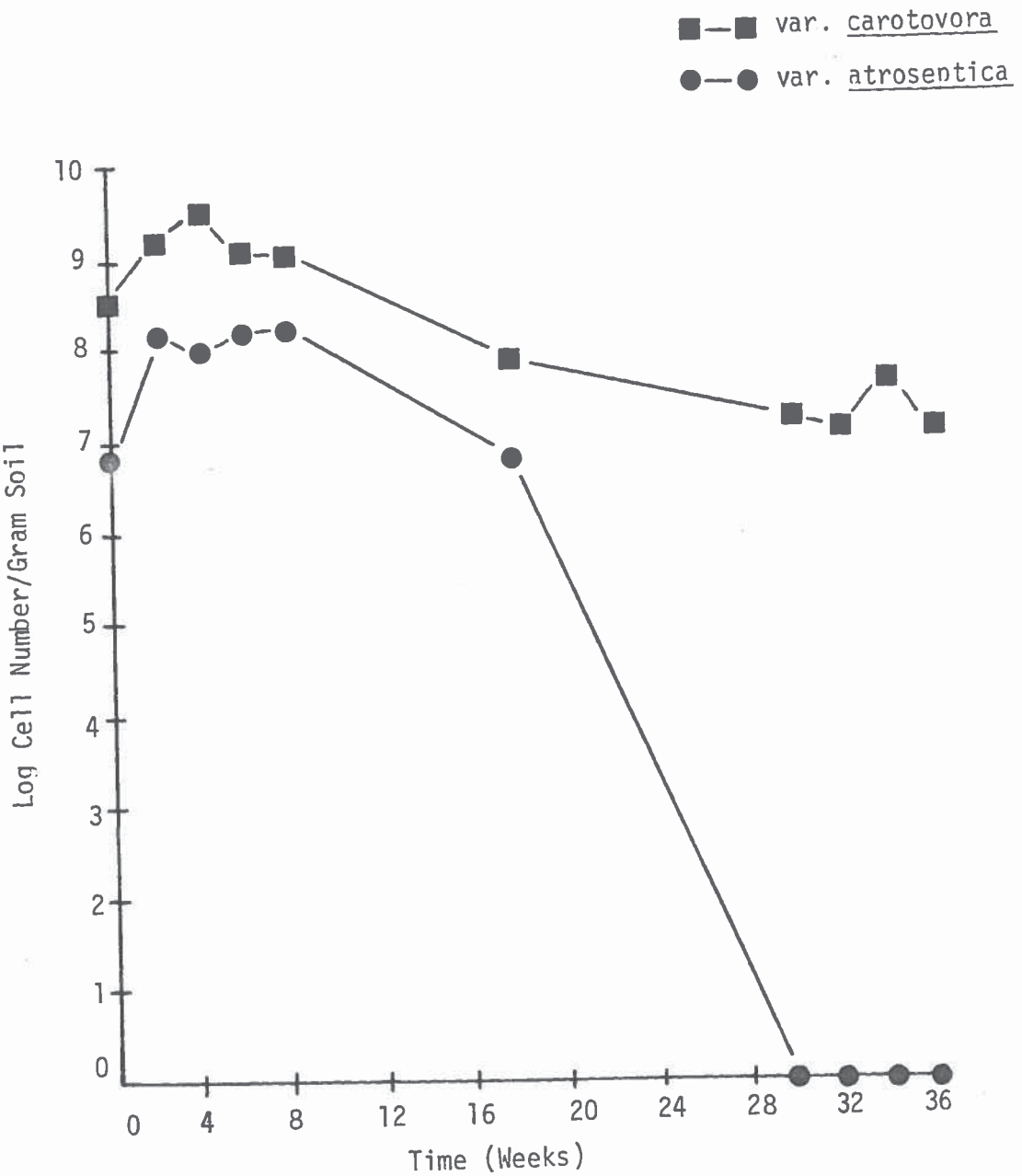
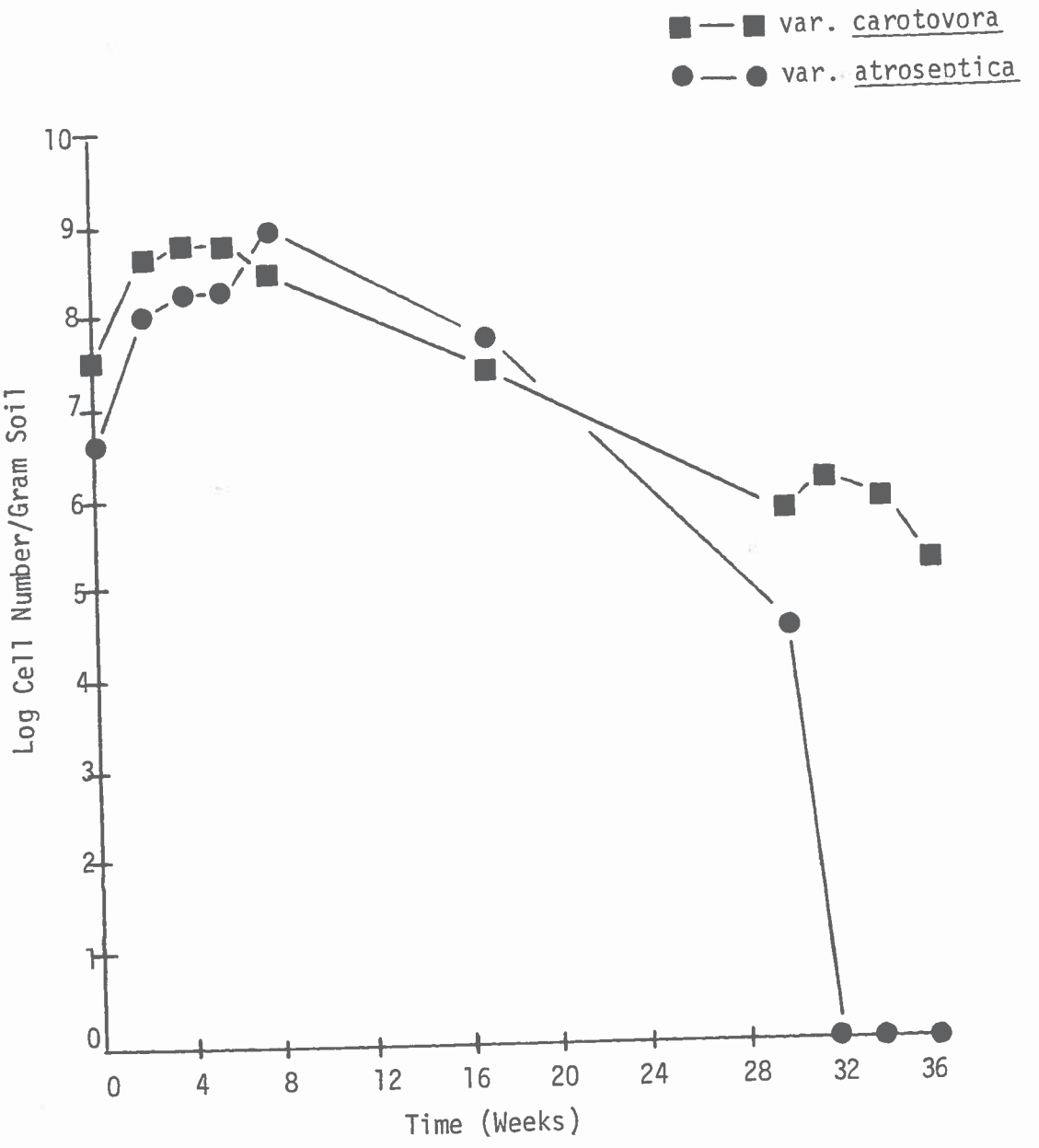


FIGURE 4. Mean numbers of Erwinia carotovora recovered from overwintered soil (September 19 to May 29).



three sampling dates. This technique permitted the recovery of cells of E. carotovora var. atroseptica and indicated that cells were present but in the low numbers, below 10^2 cells per gram of soil.

The recovery of E. carotovora var. carotovora and E. carotovora var. atroseptica from overwintered soil inoculated with bacterial suspensions of these organisms is illustrated in Figure 5 and 6, respectively. All isolates of soft rot Erwinia spp. declined more rapidly in these experiments compared to those previously described. E. carotovora var. carotovora was not recovered after 25 weeks and E. carotovora var. atroseptica after 12 weeks by direct plating onto CVP medium. The use of the anaerobic enrichment technique permitted the recovery of both isolates of E. carotovora var. carotovora for the duration of the experiments. In contrast, neither isolate of E. carotovora var. atroseptica could be recovered with enrichment culture.

The recovery of E. carotovora from buried tubers and surrounding soil at site I and site II has been tabulated in Table 7 and 8, respectively. Soft rot Erwinia spp. were recovered more frequently from the soil surrounding the tubers than the tubers themselves. Combining the results of both sites, E. carotovora var. carotovora was recovered from 14 of 50 tubers (28%) and E. carotovora var. atroseptica from 13 of 50 tubers (26%). Twenty-three of the 50 soil samples (46%) contained viable E. carotovora var. carotovora and 24 of the 50 samples (48%) harboured viable E. carotovora var. atroseptica.

FIGURE 5. Mean numbers of C-1 and C-2 isolates of Erwinia carotovora variety carotovora recovered from overwintered soil.

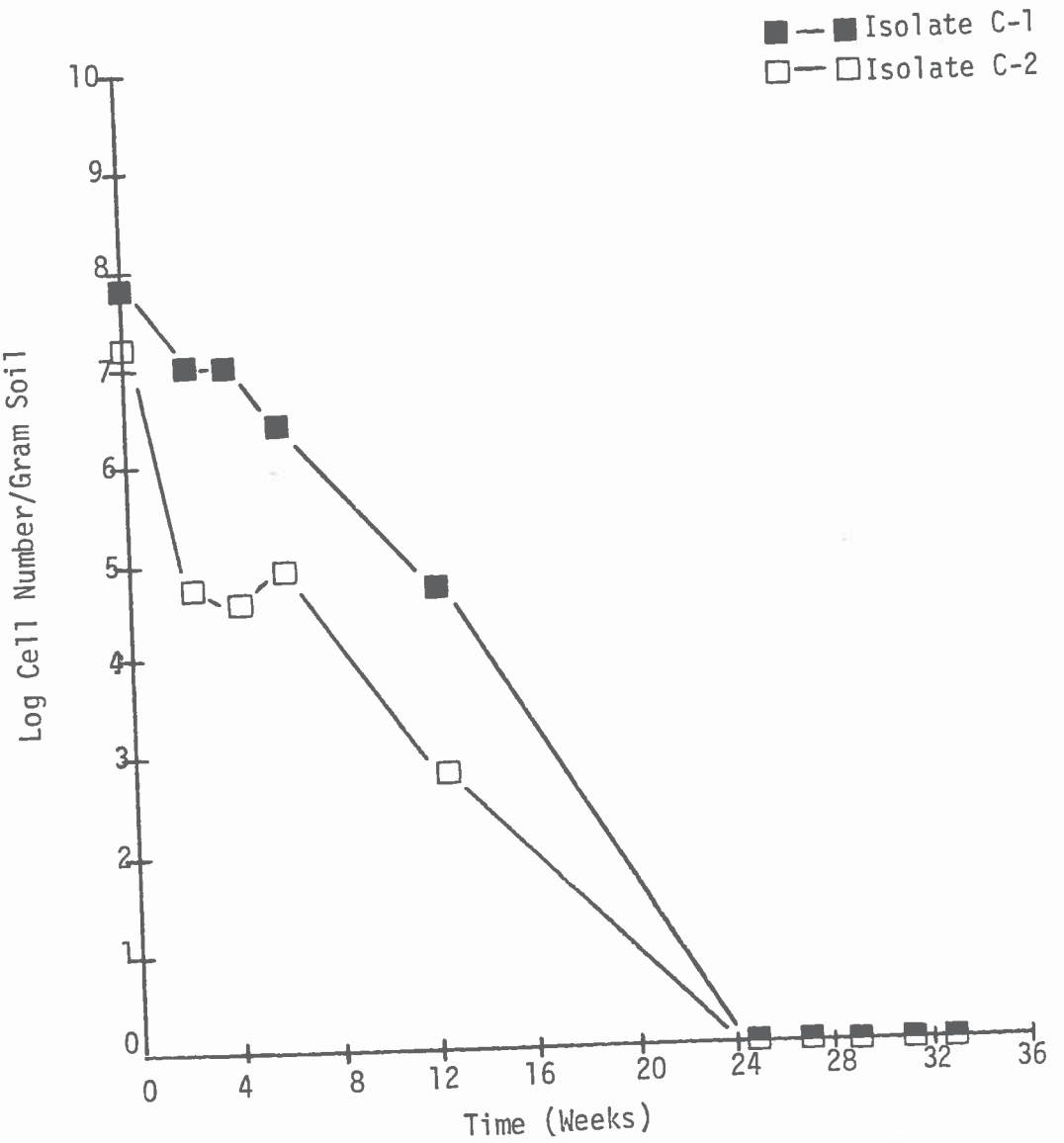


FIGURE 6. Mean numbers of A-1 and A-2 isolates of Erwinia carotovora variety atroseptica recovered from overwintered soil.

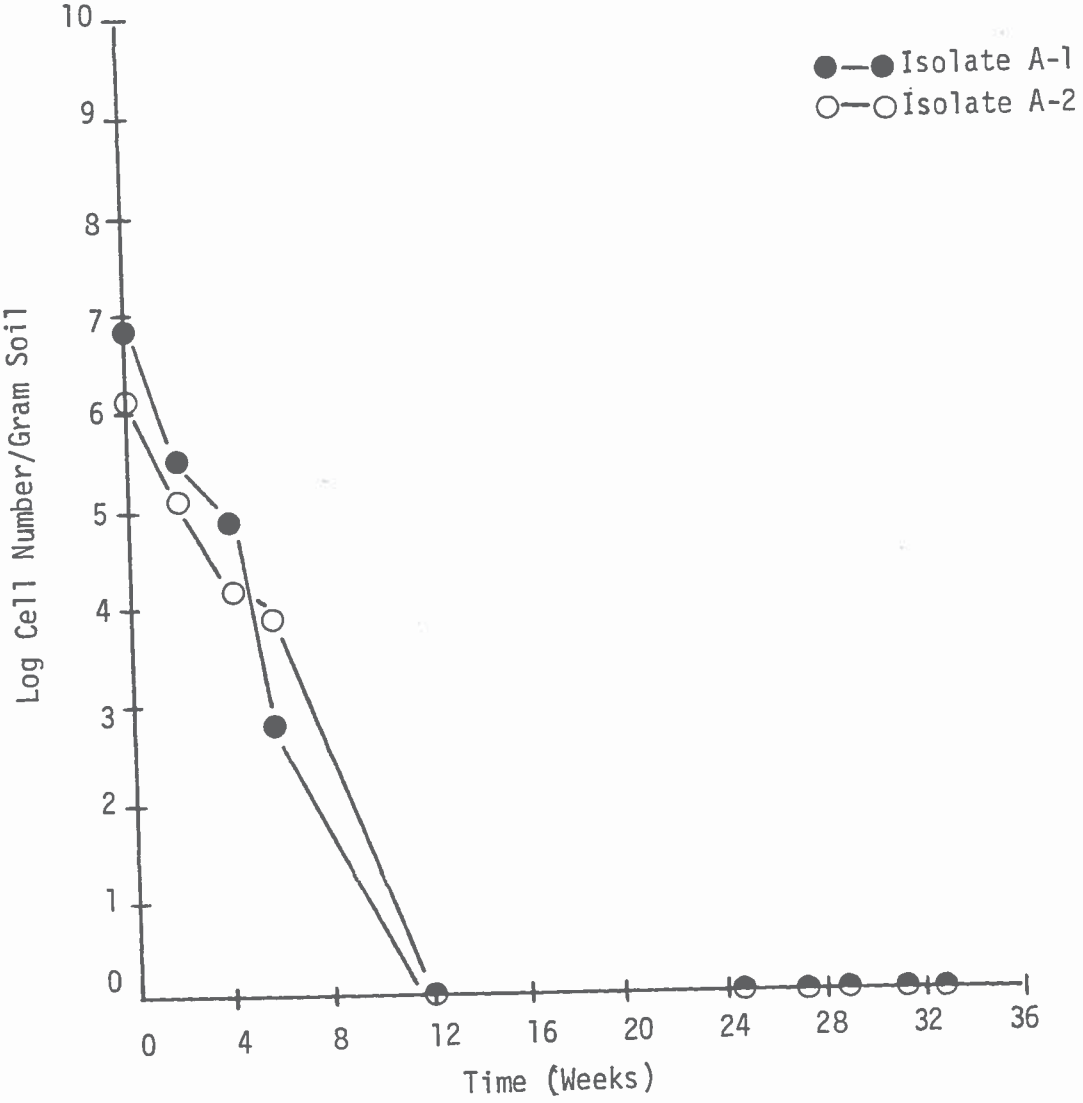


TABLE 7. *Erwinia carotovora* recovered from overwintered tubers and surrounding soil (Site I).

SAMPLING DATE	VARIETY CAROTOVORA RECOVERY				VARIETY ATROSEPTICA RECOVERY			
	TUBERS		SOIL SAMPLES		TUBERS		SOIL SAMPLES	
	No.	%	No.	%	No.	%	No.	%
April 17*	1/5	20	1/5	20	1/5	20	0/5	0
May 3	2/5	40	1/5	20	2/5	40	4/5	80
May 30	2/5	40	3/5	60	2/5	40	4/5	80
June 28	2/5	40	4/5	80	0/5	0	1/5	20
TOTAL	7/20	35	9/20	45	5/20	25	9/20	45

* Enrichment culture technique was not used at this sampling date.

TABLE 8. *Erwinia carotovora* recovered from overwintered tubers and surrounding soil (Site II).

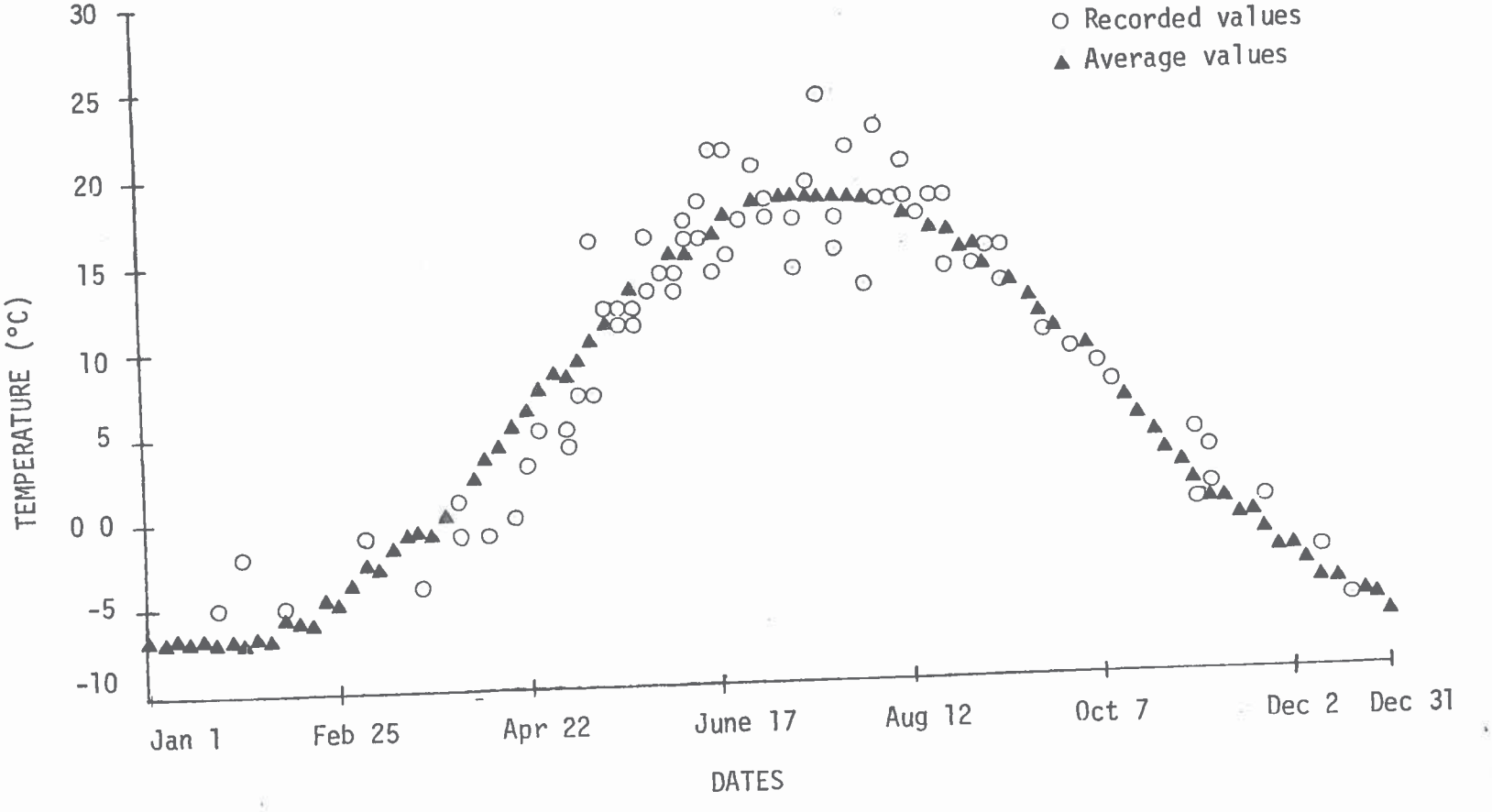
SAMPLING DATE	VARIETY *CAROTOVORA* RECOVERY				VARIETY *ATROSEPTICA* RECOVERY			
	TUBERS NO.	%	SOIL SAMPLES NO.	%	TUBERS NO.	%	SOIL SAMPLES NO.	%
May 2	3/5	60	5/5	100	2/5	40	3/5	60
May 10	0/5	0	0/5	0	3/5	60	4/5	80
May 17	2/5	40	4/5	80	1/5	20	2/5	40
May 23	2/7	29	2/7	29	1/3	33	3/3	100
June 21	0/5	0	3/5	60	1/5	20	3/5	60
June 26	0/3	0	0/3	0	0/7	0	0/7	0
TOTAL	7/30	23	14/30	47	8/30	27	15/30	50

DISCUSSION

Temperatures of +20°C, +5°C and -10°C were used in the first experiment to include the range of soil temperatures occurring under Manitoba climatic conditions. Figure 7 illustrates mean soil temperatures at 20 cm depth recorded for Glenboro sandy loam soils (Provincial Soil Testing Laboratory). The lowest soil temperatures occurred during the month of January and averaged approximately -7°C. During the latter part of July and early part of August soil temperatures were highest, averaging between 15° and 20°.

The results of the first experiment indicated that E. carotovora var. carotovora and E. carotovora var. atroseptica did not differ significantly in the length of time cells remained viable in soil. There did appear to be a significant difference in survival time at each of the three selected temperatures. Soft rot Erwinia spp. declined very rapidly at the highest temperature, +20°. Both organisms survived for longer periods at the lower temperatures, with survival greatest at +5°C. These results are in agreement with many earlier studies (Lazur and Bucur, 1964; Logan, 1968; Ficke et al., 1973; Pérombelon, 1974) on the relationship of temperature and soil survival of soft rot Erwinia spp. Studies by Logan (1968) and Ficke et al. (1973) indicated that low temperatures increased the survival time of E. carotovora in soil. High soil temperatures, greater than 20°C were reported by Pérombelon (1974) to decrease the survival time of

FIGURE 7. Average soil temperatures of Glenboro sandy loam soil at the 20 cm depth. (Provincial soil testing laboratory).



soft rot Erwinia spp. in soil.

Many of the earlier experiments undertaken to study the overwintering of soft rot Erwinia spp. in soil were based on recovering the cells by plating soil suspensions onto selective media. Many researchers (Togashi, 1972; Cuppels and Kelman, 1974; Stanghellini and Meneley, 1975; Burr and Schroth, 1977) have reported that recovery or even detection of low soil populations of soft rot Erwinia spp. is not possible with selective media. Thus, previous reports concluding that E. carotovora does not overwinter in soil may be false and due to the insensitivity of recovery techniques.

The insensitivity of CVP medium for recovering E. carotovora from soil was apparent in these experiments. In the second experiment soil inoculated with potato rot mixtures of E. carotovora overwintered under natural conditons. In early spring, E. carotovora var. carotovora was still easily recovered from the soil samples with CVP medium, but not E. carotovora var. atroseptica. An anaerobic enrichment culture technique was employed and enabled the recovery of E. carotovora var. atroseptica for the duration of the experiment. Although the latter procedure did not permit a quantitative estimate of cell populations, it indicated that low numbers of soft rot Erwinia spp. below 10^2 cells per gram of soil were still viable. Furthermore, these results support the premise that failure to detect E. carotovora in overwintered soil is due to insensitive recovery techniques and not the absence of the bacteria.

Two procedures of soil inoculation were compared in studying the overwintering of soft rot Erwinia spp. In the first method potato

rot mixtures were incorporated into the soil and this was considered to represent a more natural system. The second procedure consisted of adding bacterial suspensions to the soil and was the method frequently used in earlier studies on soil survival of E. carotovora.

The results of these experiments indicated that E. carotovora survived for longer periods of time when a potato rot mixture was used as soil inoculum. Large populations of E. carotovora var. carotovora and lesser numbers of E. carotovora var. atroseptica were still recovered in early spring. Both varieties of E. carotovora declined very rapidly when bacterial suspensions were used as the source of inoculum. There were no traces of viable E. carotovora var. atroseptica and only low populations of E. carotovora var. carotovora in early spring.

Implications that E. carotovora survives for longer periods when in association with plant tissue are reflected in these results. Furthermore, these findings agree with earlier reports (Logan, 1969; Ficke et al., 1973; Copeman and Schneider, 1975; Vorokovich, 1960; Burr and Schroth, 1977) that association with plant debris or the roots of a diverse number of plants lengthens the survival time of E. carotovora. Presumably, living plant tissue and the debris of dead plants provide more favourable loci for bacteria than does the soil matrix (Schuster and Coyne, 1974). This implication has been verified by the results of Burr and Schroth (1977). E. carotovora was detected in field soil where vegetation was present or in harvested fields where plant debris was visible. In contrast, there were no traces of Erwinia in fallow soils devoid of vegetation or recognizable plant residues.

The results of the final experiment indicated that E. carotovora overwintered in buried, soft rotting tubers and surrounding soil and

could possibly serve as a source of inoculum in the spring. These results contradict the previous discussion in that E. carotovora was recovered more frequently from the soil surrounding the tubers than the tubers themselves. One possible explanation for the loss of viability within the tubers is the antagonistic effect of other microorganisms. This observation has been reported by Logan (1969) who found that viability of Erwinia within tubers was reduced once the tissue had been colonized by secondary invaders. Furthermore, E. carotovora was reported to survive for longer periods in slightly infected rather than well rotted tubers (Logan, 1969).

During the initial recovery of Erwinia from overwintered tubers and surrounding soil, a large number of Pseudomonas spp. colonies overcrowded the CVP medium and made it more difficult to detect Erwinia colonies on the plates. Consequently, an anaerobic enrichment technique was used which favoured the build-up of Erwinia and eliminated the growth of aerobic Pseudomonas spp. The anaerobic enrichment technique enabled the recovery of soft rot Erwinia spp. from tubers and soil at both sites and indicated that antagonism by Pseudomonas spp. may be of great significance. This observation agrees with earlier reports by Logan (1969) and Mew et al. (1976) that certain microflora may influence the infection potential and viability of Erwinia.

The survival of E. carotovora var. carotovora and E. carotovora var. atroseptica in soil did not appear to differ significantly under controlled laboratory conditions. Succeeding experiments in which soil was incubated under natural conditions indicated that E. carotovora var. carotovora survived in greater numbers and for longer periods.

The more frequent recovery of E. carotovora var. carotovora from field and rhizosphere soil (Meneley and Stanghellini, 1976; Burr and Schroth, 1977) indicates that this organism may be better adapted to soil survival. Furthermore, this could account for the frequent recontamination of virus tested stem cutting stocks by E. carotovora var. carotovora (Pérombelon, 1974; Pérombelon and Lowe, 1976; Molina and Harrison, 1977) including the first year of multiplication of the cuttings. More research is needed in this area to evaluate the respective roles of E. carotovora var. carotovora and E. carotovora var. atroseptica in the development of blackleg.

PART THREE

The effect of low temperature on the *in vitro* growth of *Erwinia carotovora*

INTRODUCTION

It has been well established (Bonde, 1950; Lazur and Bucur, 1964) that temperature is an important factor in the development of blackleg and soft rot. The effect of temperature on pathogenicity (Burkholder and Smith, 1949; Hellmers and Dowson, 1953; Graham and Dowson, 1960a), tuber break down (Davidson, 1948; Murant and Wood, 1957; Logan, 1969; Pérombelon and Lowe, 1972c), and soil survival (Logan, 1969; Ficke *et al.*, 1973a; Pérombelon, 1974) have been studied extensively. Few studies (Lazur and Bucur, 1964; Erinle, 1975) have been undertaken on the growth, either *in vivo* or *in vitro* of *E. carotovora*.

Therefore, the present study was conducted to determine the effect of low temperatures on the growth of *E. carotovora* var. *carotovora* and *E. carotovora* var. *atrosetpica*.

MATERIALS AND METHODS

Selection of isolates:

Eleven isolates of *E. carotovora* var. *carotovora* and eleven of *E. carotovora* var. *atroseptica* were chosen for this study. The origin of each isolate is listed in Table 9.

Procedure for measuring growth:

Single colonies from 48-72 hour nutrient agar plates were suspended in 10 ml of nutrient broth contained in screw-capped culture tubes. The tubes were incubated on a shaker tray for 16 hours at room temperature (approximately 25°C) for cultures to reach the exponential growth phase.

One ml of inoculum was added to each of three sterile Bausch and Lomb spectrophotometer tubes (three replicates per isolate) containing four ml of sterile nutrient broth. The tubes were fitted with sterile cotton plugs to keep the broth aerated and free from contamination. The tubes were incubated at 10°C, 15°C and 20°C by adjusting the water temperature of a thermo-shake bath. Three replicates per isolate per temperature were used in this study.

Turbidity was measured by taking absorbance readings on a Bausch and Lomb Spectrophotometer 20 set at 540 nm. Initial readings were taken at time zero and then at two hour (15° and 20°) and four hour (10°) intervals for a period of six and twelve hours respectively.

Absorbance readings were converted to cell numbers (Appendix III).

TABLE 9. Origin of Erwinia carotovora isolates used in growth studies.

Erwinia carotovora var. atroseptica		Erwinia carotovora var. carotovora	
Isolate	Origin	Isolate	Origin
A-1	Manitoba Blackleg Plant-1976	C-1	Manitoba Soft Rot Tuber-1976
A-2	Manitoba Blackleg Plant-1977	C-2	Manitoba Soft Rot Tuber-1977
A-3	Manitoba Soft Rot Tuber-1977	C-3	Manitoba Soft Rot Tuber-1977
A-4	Manitoba Soft Rot Tuber-1977	C-4	Manitoba Soft Rot Tuber-1977
A-5	Manitoba Soft Rot Tuber-1977	C-5	Manitoba Soft Rot Tuber-1977
A-6	Manitoba Soft Rot Tuber-1977	C-6	Manitoba Soft Rot Tuber-1977
A-7	Manitoba Soft Rot Tuber-1977	C-7	Manitoba Soft Rot Tuber-1977
A-8	Manitoba Soft Rot Tuber-1977	C-8	Manitoba Soft Rot Tuber-1977
A-9	Scotland	C-9	Scotland
A-10	British Columbia	C-10	Arizona
A-11	Wisconsin	C-11	British Columbia

Linear regression analysis based on three replicates per isolate was used to compute growth rates. Mean generation times of isolates at 10^{o}, 15^{o} and $20^{o}C$ were calculated from the following equation: $T = \ln 2 / k$ where T = mean generation time; k = specific growth rate (Dawes, 1969).

RESULTS AND DISCUSSION

Table 10 and 11 illustrate the mean generation time of eleven isolates of E. carotovora var. atroseptica and E. carotovora var. carotovora, respectively, at 10°, 15° and 20°C. Generation times decreased as the incubation temperatures increased for both varieties of E. carotovora.

Figure 8 illustrates the mean generation time of all isolates of E. carotovora var. carotovora and E. carotovora var. atroseptica. There were no significant (P=0.1) differences between these organisms at 15°C and 20°C whereas at 10°C E. carotovora var. atroseptica grew significantly faster.

Variation in growth rate among isolates of E. carotovora var. atroseptica and E. carotovora var. carotovora is illustrated in Table 12 and 13, respectively. The greatest variation among isolates occurred at 10°C with generation times ranging from 6.8 to 12.0 hours for isolates of E. carotovora var. atrosetpica and 7.7 to 13.8 hours for those of E. carotovora var. carotovora. There were no significant (P=.01) differences in growth rate among isolates at 15°C. However, at 20°C, isolates of E. carotovora var. carotovora differed significantly (P=.01) whereas isolates of E. carotovora var. atroseptica did not.

There did not appear to be any correlation between growth rate at these temperatures and geographic location. Manitoba isolates of E. carotovora var. atroseptica and E. carotovora var. carotovora were distributed among the slowest growing and fastest growing isolates at

TABLE 10. Mean generation time of eleven isolates of Erwinia carotovora variety atroseptica at 10°C, 15°C and 20°C.

Isolate	Mean generation time (hours) Temperature 10°	15°	20°
A-1	6.8* (1)**	4.0 (2)	3.9 (10)
A-2	7.6 (2)	5.2 (11)	3.4 (6)
A-3	8.7 (5)	4.7 (10)	3.0 (2)
A-4	9.2 (6)	4.4 (7)	3.7 (9)
A-5	12.0 (11)	3.9 (1)	3.7 (8)
A-6	10.1 (10)	4.3 (5)	4.0 (11)
A-7	9.4 (9)	4.4 (6)	3.5 (7)
A-8	9.3 (7)	4.1 (3)	3.0 (3)
A-9	7.6 (3)	4.1 (4)	3.0 (4)
A-10	8.5 (4)	4.6 (9)	2.9 (1)
A-11	9.4 (8)	4.4 (8)	3.3 (5)

* Mean of three replicates.

** The number in parenthesis indicates the ranking of each isolate at each temperature in order of increasing generation times.

TABLE 11. Mean generation time of eleven isolates of Erwinia carotovora variety carotovora at 10°C, 15°C and 20°C.

Isolate	Mean generation time (hours) Temperature 10°	15°	20°
C-1	11.6* (9)**	4.6 (8)	3.6 (8)
C-2	9.7 (4)	5.3 (11)	4.5 (11)
C-3	7.7 (1)	4.3 (5)	4.3 (10)
C-4	13.8 (11)	3.9 (1)	4.2 (9)
C-5	9.9 (5)	3.9 (2)	2.9 (3)
C-6	9.6 (3)	4.1 (4)	3.1 (6)
C-7	9.1 (2)	4.5 (7)	3.6 (7)
C-8	12.4 (10)	5.2 (10)	3.0 (5)
C-9	10.8 (7)	4.9 (9)	2.5 (1)
C-10	10.8 (6)	4.1 (3)	2.6 (2)
C-11	11.4 (8)	4.5 (6)	2.9 (4)

* Mean of three replicates.

** The number in parenthesis indicates the ranking of each isolate at each temperature in order of increasing generation times.

FIGURE 8. Mean generation time of eleven isolates of Erwinia carotovora variety atroseptica and Erwinia carotovora variety carotovora at +10°C, +15°C and +20°C.

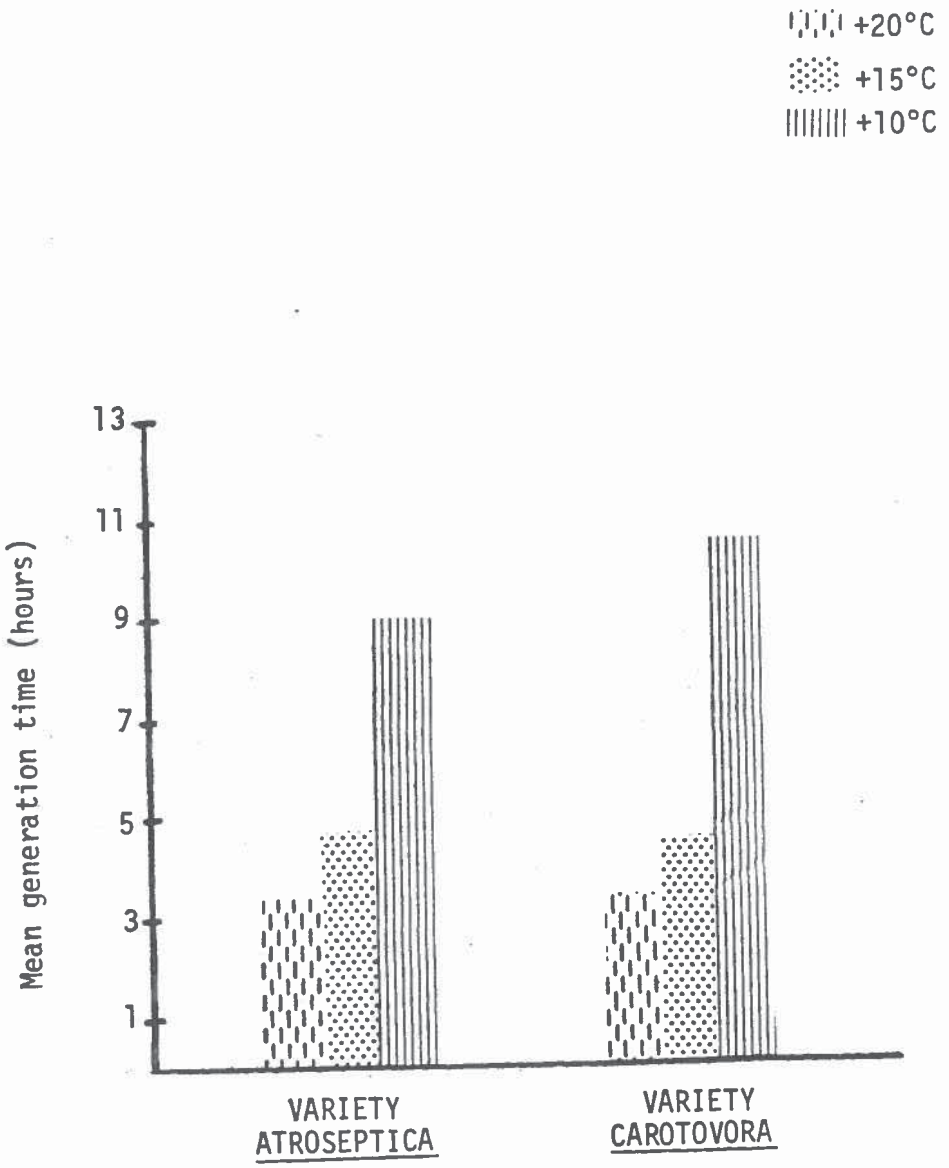


TABLE 12. Mean generation time of eleven isolates of Erwinia carotovora variety atroseptica at 10°C, 15°C and 20°C.

Mean generation time (hours)					
Temperature					
10°C		15°C		20°C	
Isolate		Isolate		Isolate	
A-1	6.8* a**	A-5	3.9 a	A-10	2.9 a
A-2	7.6 ab	A-1	4.0 a	A-3	3.0 a
A-9	7.6 ab	A-8	4.1 a	A-8	3.0 a
A-10	8.5 bc	A-9	4.1 a	A-9	3.0 a
A-3	8.7 bc	A-6	4.3 a	A-11	3.3 a
A-4	9.2 bc	A-7	4.4 a	A-2	3.4 a
A-8	9.3 c	A-4	4.4 a	A-7	3.5 a
A-11	9.4 c	A-11	4.4 a	A-5	3.7 a
A-7	9.4 c	A-10	4.6 a	A-4	3.7 a
A-6	10.1 c	A-3	4.7 a	A-1	3.9 a
A-5	12.0 d	A-2	5.2 a	A-6	4.0 a

* Mean of three replicates.

** Values within columns followed by the same letter are not significantly (P=0.01) different by Tukey's test of ordered means.

TABLE 13. Mean generation time of eleven isolates of Erwinia carotovora variety carotovora at 10°C, 15°C and 20°C.

Mean generation time					
Temperature					
10°C		15°C		20°C	
Isolate		Isolate		Isolate	
C-3	7.7* a**	C-4	3.9 a	C-9	2.5 a
C-7	9.1 ab	C-5	3.9 a	C-10	2.6 ab
C-6	9.6 bc	C-10	4.1 a	C-5	2.9 abc
C-2	9.7 bcd	C-6	4.1 a	C-11	2.9 abc
C-5	9.9 bcde	C-3	4.3 a	C-8	3.0 abc
C-10	10.8 cdef	C-11	4.5 a	C-6	3.1 abc
C-9	10.8 cdefg	C-7	4.5 a	C-7	3.6 abc
C-11	11.4 efg	C-1	4.6 a	C-1	3.6 abc
C-1	11.6 fg	C-9	4.9 a	C-4	4.2 bc
C-8	12.4 gh	C-8	5.2 a	C-3	4.3 c
C-4	13.8 h	C-2	5.3 a	C-2	4.5 c

* Mean of three replicates.

** Values within columns followed by the same letter are not significantly (P=0.01) different by Tukey's test of ordered means.

each of the three selected temperatures (Table 10 and 11).

Growth experiments *in vitro* may not accurately reflect the growth of a pathogenic bacterium in soil and plant tissue under natural conditions where the supply of nutrients is in a continually dynamic state. Also, diurnal changes can affect both host and pathogen together or independently. The experiments described here have a further practical limitation in that the inocula used throughout were cultures grown at 25°C, regardless of the subsequent incubation temperature. However, the main purpose of this study was to compare the growth response of isolates of *E. carotovora* var. *carotovora* and *E. carotovora* var. *atroseptica* at three selected temperatures and all isolates received similar treatment prior to testing.

Erinle (1975) has studied the growth *in vivo* of *E. carotovora*. He reported that the growth of *E. carotovora*, especially variety *carotovora* was retarded at lower daily maximum temperatures (c. 17°C). Similarly, the results of this study indicated that at 10°C, isolates of *E. carotovora* var. *carotovora* had a higher mean generation time and, therefore, grew more slowly than isolates of *E. carotovora* var. *atroseptica*. However, some isolates of *E. carotovora* var. *carotovora* did have considerably shorter mean generation times than others at 10°C and might be expected to be active at such a temperature in the field.

Reports by Molina and Harrison (1977) have indicated that the occurrence of *E. carotovora* var. *carotovora* associated with stem infections appears to be greatly influenced by geographic location. Furthermore, the higher temperature appeared to favour *E. carotovora* var. *carotovora* since it was isolated nearly three times more frequently from the warmer area (Molina and Harrison, 1977). In contrast, the data in

this study indicated that mean generation times for isolates of *E. carotovora* var. *carotovora* and *E. carotovora* var. *atroseptica* did not differ significantly at 15° and 20° and that there did not appear to be any correlation between growth rate at these particular temperatures and geographic location.

GENERAL DISCUSSION

This study established that both varieties of E. carotovora are responsible for causing blackleg and soft rot in Manitoba. E. carotovora var. carotovora is mainly responsible for tuber breakdown in storage whereas E. carotovora var. atroseptica is the main causal agent of blackleg infections in the field. However, the isolation of E. carotovora var. carotovora from cuttings of nuclear stock plants and occasionally from infected plants in the field indicates that this organism may be important in causing blackleg under certain environmental conditons.

More research is needed in assessing the respective roles of both varieties of E. carotovora in causing blackleg. The previous lack of awareness of the involvement of E. carotovora var. carotovora has hampered the evaluation of recent control techniques involving the stem cutting procedure. E. carotovora var. carotovora has been reported by many researchers (Graham and Hardie, 1971; Pérombelon, 1974; Pérombelon and Lowe, 1976; Molina and Harrison, 1977) to be the main organism responsible for recontamination of virus-tested stem cutting (VTSC) stocks. The significance of this recontamination needs to be studied more thoroughly.

E. carotovora var. carotovora and E. carotovora var. atroseptica are capable of surviving in soil over winter in Manitoba. Survival in soil is prolonged when the cells are in close association with plant debris. Earlier reports indicating poor survival of E. carotovora in soil may have resulted from insensitive techniques used in recovering the

bacteria. Detection of E. carotovora in soil, especially E. carotovora var. carotovora has been reported by Meneley and Stanghellini (1976) and Burr and Schroth (1977). Additional research is needed to evaluate the importance of viable E. carotovora in soil as a source of inoculum in the spring. Several different inoculum sources for recontamination appear to be involved and all must be identified and eliminated before blackleg-tested seed production will be feasible.

There were no significant differences in the mean generation time between E. carotovora var. carotovora and E. carotovora var. atroseptica at temperatures of 15° and 20°C whereas at 10°C mean generation times differed significantly. At this temperature isolates of E. carotovora var. atroseptica grew significantly faster than isolates of E. carotovora var. carotovora.

Variability among isolates of E. carotovora var. carotovora and E. carotovora var. atroseptica was greatest at 10°C. At 15°C there were no significant differences among isolates of either variety of E. carotovora and at 20°C isolates of E. carotovora var. carotovora differed significantly whereas isolates of E. carotovora var. atroseptica did not differ significantly in growth rate.

There did not appear to be any correlation between growth rate at these selected temperatures and geographic location. Manitoba isolates of E. carotovora were distributed among the slowest growing and fastest growing isolates at all temperatures.

It is evident that more research is needed to study the effects of temperature on the growth, in vitro and in vivo of E. carotovora. The results of these studies may reveal differences and/or similarities between E. carotovora var. carotovora and E. carotovora var. atroseptica

which could prove significant in the development of blackleg in the field.

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APPENDIXES

APPENDIX I

PREPARATION OF MEDIA

(A) Crystal Violet Pectate: (Cuppels and Kelman, 1971)

Ingredients:

4.5 ml	1N NaOH
3.0 ml	10% $CaCl_2.2\ H_2O$
1.5 g	Difco agar
1.0 g	$NaNO_3$
15.0 g	Sodium polypectate
1.0 ml	0.075% (W/V) Crystal violet solution
500 ml	distilled water

Preparation: Preheat a five-cup Waring blender with running hot tap water or rinse in boiling water. Place 300 ml boiling distilled water in the blender plus the first four ingredients. Blend at high speed for 15 seconds. Slowly add 15 g sodium polypectate (Orange Products Division, Sunkist Growers, Ontario, California). Blend for 15 seconds and gradually add 200 ml boiling distilled water. Place in a 2 litre flask and cap with aluminum foil rather than a cotton plug. Autoclave for 25 minutes at 120°C and 15 lb/sq in. steam pressure. Allow pressure to drop slowly to avoid bubbles. Add one ml 0.075% crystal violet solution to the hot medium and pour plates as soon as possible.

(B) PT Broth: (Burr and Schroth, 1977)

Ingredients:

5.0 g	Polygalacturonic acid
1.0 g	$NaNO_3$
4.0 g	K_2HPO_4
.2 g	$MgSO_4$
.1 ml	Tergitol
17.0 ml	1N NaOH
1.0 l	distilled water

Preparation: Dissolve all the ingredients in water. Pour into bottles, cap and sterilize 15 minutes at 121°C and 15 lbs/sq. in. steam pressure.

(C) Potato broth:

Ingredients:

400 g	peeled, cubed potato tubers
10 g	dextrose
600 ml	distilled water

Preparation: Peeled and cubed potato tubers were steamed for one hour in 600ml of distilled water. About 500 ml of extract was decanted and dispensed in 1,000 ml erlenmeyer flasks. Ten grams of dextrose were added to each 500 ml of extract. The flasks were fitted with cotton plugs and the broth autoclaved for 15 minutes at 121°C and 15 lb/sq. in. steam pressure.

APPENDIX II

(a) Analysis of variance

Growth of eleven isolates of Erwinia carotovora variety carotovora and Erwinia carotovora variety atroseptica at three temperatures.

Source of Variation	D.F.	S.S.	M.S.	F.
Temperature	2	519.0665	259.5333	267.28**
Variety	1	5.5739	5.5739	5.74*
T x V	2	9.5857	4.7929	4.94*
Error	60	58.2593	.9710	
Total	64	592.4854		

** Significant at the 1% level.

* Significant at the 5% level.

(b) Analysis of variance

Mean numbers of Erwinia carotovora incubated at various temperatures (+20°C, +5°C and -10°C).

Source	D.F.	S.S.	M.S.	F.
Replications	2	25.854	12.927	2.93 NS
Main plots (time)	6	538.667	89.778	20.38 **
Main plot error	12	52.876	4.406	
Bacteria	1	4.927	4.927	2.29 NS
Temperature	2	349.281	174.641	81.04 **
Bact. x Temp.	2	11.946	5.973	2.77 NS
Time x Bacteria	6	5.544	0.924	.43 NS
Time x Temp.	12	129.223	10.769	5.00 **
Time x Bact. x Temp.	12	15.892	1.324	.61 NS
Subplot error	70	150.860	2.155	
Total	125	1285.072		

** Significant at the 1% level

* Significant at the 5% level

NS Not significant

APPENDIX III

PROCEDURE FOR CORRELATING ABSORBANCE READINGS WITH BACTERIAL CELL NUMBERS

A nutrient agar slant was streaked with E. carotovora var. atroseptica and incubated for 24 hours at 25°C. Bacterial growth was suspended in five ml of buffered water and one ml was added to 100 ml of nutrient broth contained in a sterile, Erlenmeyer flask. The flask was fitted with a sterile, cotton plug and incubated on a shaker tray for 16 hours at room temperature. A series of tubes with increasing amounts of inoculum was set up as follows:

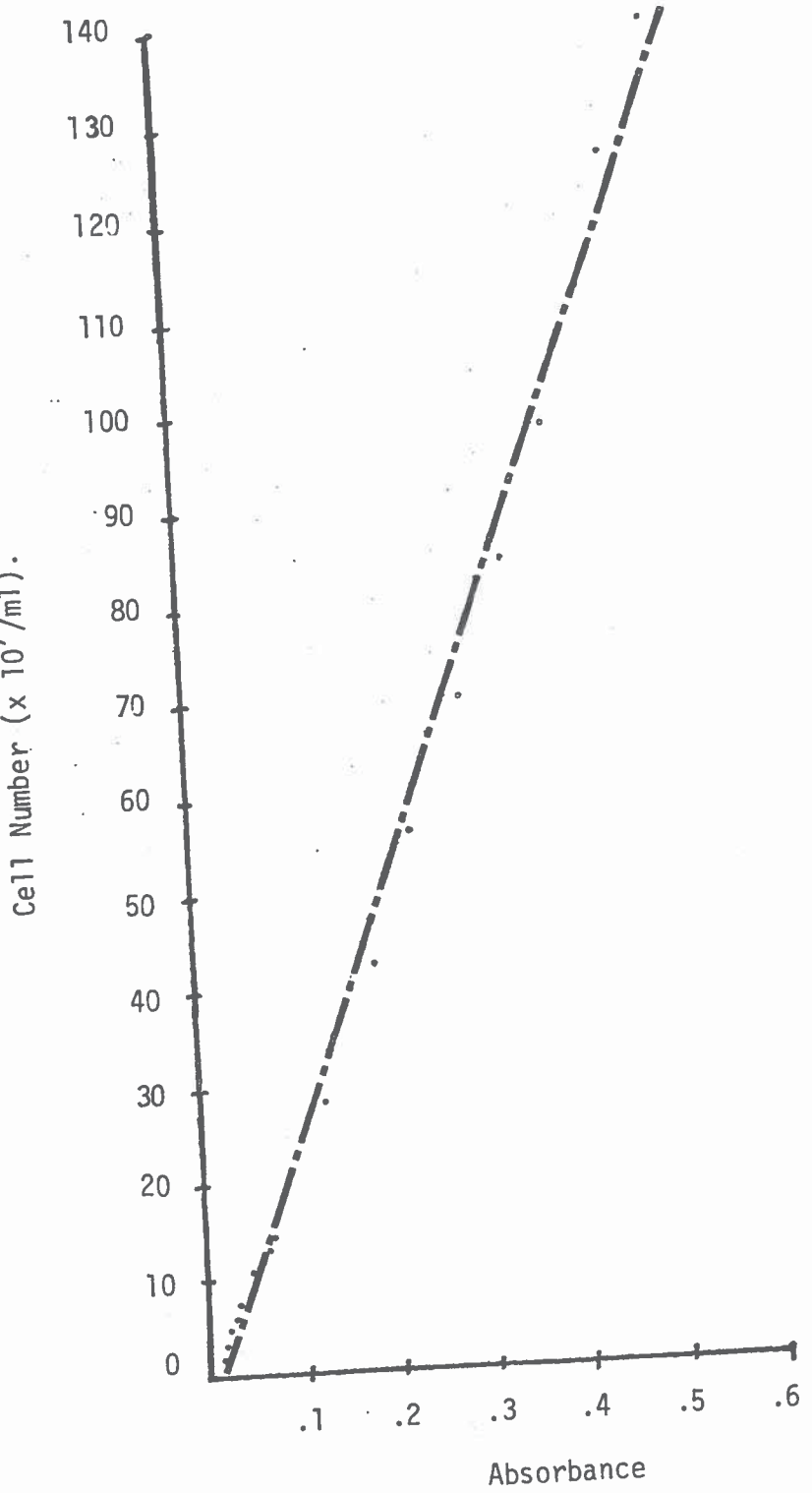
Tube No.	Inoculum(ml)	Diluent(ml)
1	.05	4.95
2	.10	4.90
3	.15	4.85
4	.20	4.80
5	.25	4.75
6	.30	4.70
7	.35	4.65
8	.40	4.60
9	.45	4.55
10	.50	4.50
11	1.00	4.00
12	1.50	3.50
13	2.00	3.00
14	2.50	2.50
15	3.00	2.00
16	3.50	1.50
17	4.00	1.00
18	4.50	0.50
19	5.00	0.00

Turbidity of the suspensions in these nineteen tubes was measured by taking absorbance readings at 530 nm with a Bausch and Lomb Spectrophotometer 20.

Four one-ml aliquots of the original inoculum in the exponential growth phase were removed from the flask and serially diluted. Then, 0.1 ml aliquots of each dilution (1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}) were plated on nutrient agar and the plates incubated at $25^{\circ}C$.

Plates were counted after a 48 hour incubation period and the mean number of cells per ml of inoculum (four replicates) was calculated. The number of cells per ml of suspension was then calculated for each of the nineteen tubes. Absorbance readings (taken 48 hours previously) were plotted against cell concentration yielding a linear relationship (Figure 1). The straight line is represented by the equation: $Y = -3.7672 + 269.7412(X)$.

FIGURE 1. Conversion of absorbance readings to cell numbers.



Appendix IV

TABLE 1. Mean numbers of Erwinia carotovora incubated at various temperatures (+20°C, +5°C and -10°C).

TIME	LOG (CELLS PER GRAM SOIL)					
	Variety atroseptica			Variety carotovora		
	Temperature			Temperature		
(WEEKS)	+20°	+5°	-10°	+20°	+5°	-10°
0	7.30	8.93	8.26	7.87	9.10	7.87
1	7.03	8.48	7.39	7.65	8.87	8.65
2	6.59	8.52	6.16	5.43	7.97	8.55
3	4.87	8.03	5.59	3.77	7.69	6.59
6	NR*	7.63	5.03	NR	7.83	6.47
9	2.67	6.69	3.77	NR	6.88	5.20
12	NR	4.87	2.67	NR	6.69	3.67

* No recovery

TABLE 2. Erwinia carotovora variety carotovora recovered from overwintered soil (September 12 to May 22).

TIME	Cell number per gram soil			
	Replicate			Mean
Weeks	A	B	C	
0	3.5×10^{8}[a]	2.5×10^{8}	1.5×10^{8}	2.5×10^{8}
2	1.2×10^{9}	2.0×10^{9}	2.0×10^{9}	1.7×10^{9}
4	4.7×10^{9}	3.2×10^{9}	1.9×10^{9}	3.3×10^{9}
6	6.0×10^{8}	2.4×10^{9}	1.2×10^{9}	1.4×10^{9}
8	2.1×10^{9}	6.0×10^{8}	1.3×10^{9}	1.3×10^{9}
18	8.0×10^{6}	1.2×10^{8}	1.2×10^{8}	8.3×10^{7}
30	1.8×10^{7}	3.9×10^{7}	6.0×10^{6}	2.1×10^{7}
32	1.9×10^{7}	1.2×10^{7}	1.1×10^{7}	1.4×10^{7}
34	5.7×10^{7}	2.6×10^{7}	7.9×10^{7}	5.4×10^{7}
36	1.8×10^{7}	1.3×10^{5}	2.8×10^{7}	1.5×10^{7}

[a] Selective medium used; lower limit of recovery is 10^{2} cells per gram soil.

TABLE 3. Erwinia carotovora variety atroseptica recovered from overwintered soil (September 12 to May 22).

Time	Cell number per gram soil			
	Replicate			Mean
WEEKS	A	B	C	
0	1.3×10^{7}[a]	7.0×10^{6}	1.9×10^{6}	7.0×10^{6}
2	1.8×10^{8}	2.5×10^{8}	5.5×10^{7}	1.6×10^{8}
4	5.4×10^{7}	1.4×10^{8}	1.4×10^{8}	1.1×10^{8}
6	3.0×10^{8}	8.0×10^{7}	1.2×10^{8}	1.7×10^{8}
8	2.5×10^{7}	3.0×10^{8}	1.7×10^{8}	1.7×10^{8}
18	8.5×10^{7}	4.7×10^{7}	5.5×10^{7}	6.3×10^{7}
30	NR[b]	NR	NR	
32	$R^{E}(1.7 \times 10^{8})$[c]	$R^{E}(7.0 \times 10^{6})$	$R^{E}(1.2 \times 10^{8})$	(9.9×10^{7})
34	$R^{E}(4.9 \times 10^{7})$	$R^{E}(3.2 \times 10^{7})$	$R^{E}(2.6 \times 10^{7})$	(3.6×10^{7})
36	$R^{E}(1.5 \times 10^{7})$	$R^{E}(1.6 \times 10^{7})$	$R^{E}(1.3 \times 10^{7})$	(1.5×10^{7})

[a] Selective medium used; lower limit of recovery is 10^{2} cells per gram soil.

[b] Not recovered with selective medium.

[c] Recovered with enrichment culture technique; values in parenthesis do not represent actual numbers.

TABLE 4. Erwinia carotovora variety carotovora recovered from overwintered soil (September 19 to May 29).

Time	Cell number per gram soil			
	Replicate			Mean
Weeks	A	B	C	
0	4.7×10^{7}[a]	2.4×10^{7}	2.7×10^{7}	3.3×10^{7}
2	4.7×10^{8}	3.7×10^{8}	2.4×10^{8}	3.6×10^{8}
4	1.9×10^{8}	8.0×10^{7}	1.8×10^{9}	6.9×10^{8}
6	3.6×10^{7}	7.5×10^{7}	8.1×10^{7}	6.4×10^{8}
8	3.9×10^{8}	2.0×10^{8}	2.4×10^{8}	2.8×10^{8}
17	2.8×10^{7}	1.4×10^{7}	5.0×10^{7}	3.0×10^{7}
30	3.5×10^{5}	2.7×10^{5}	1.2×10^{6}	6.0×10^{5}
32	1.0×10^{6}	1.5×10^{6}	1.4×10^{6}	1.3×10^{6}
34	1.1×10^{6}	4.0×10^{5}	1.5×10^{6}	1.0×10^{6}
36	2.5×10^{5}	2.0×10^{5}	6.0×10^{4}	1.7×10^{5}

[a] Selective medium used; lower limit of recovery is 10^{2} Cells per gram soil.

TABLE 5. Erwinia carotovora variety atroseptica recovered from overwintered soil (September 19 to May 29).

Time	Cell number per gram soil			
	Replicate			Mean
Weeks	A	B	C	
0	5.0×10^{6}[a]	1.0×10^{6}	5.5×10^{6}	3.8×10^{6}
2	3.0×10^{8}	3.8×10^{7}	2.3×10^{6}	1.1×10^{8}
4	2.1×10^{8}	9.0×10^{7}	1.8×10^{8}	1.6×10^{8}
6	3.2×10^{8}	1.0×10^{8}	2.7×10^{7}	1.5×10^{8}
8	8.8×10^{8}	4.5×10^{8}	8.3×10^{8}	7.2×10^{8}
17	1.5×10^{7}	1.3×10^{8}	1.8×10^{7}	5.4×10^{7}
30	7.0×10^{4}	4.0×10^{4}	2.0×10^{4}	4.3×10^{4}
32	NR[b]	NR	NR	
34	$R^{E}(2.3 \times 10^{6})$[c]	$R^{E}(5.0 \times 10^{3})$	$R^{E}(1.0 \times 10^{4})$	(7.7×10^{5})
36	$R^{E}(8.6 \times 10^{4})$	$R^{E}(5.6 \times 10^{4})$	$R^{E}(2.6 \times 10^{5})$	(1.4×10^{5})

[a] Selective medium used; lower limit of recovery is 10^{2} cells per gram soil.

[b] Not recovered with selective medium.

[c] Recovered with enrichment culture technique; values in parenthesis do not represent actual numbers.

TABLE 6. C-1 isolate of *Erwinia carotovora* variety *carotovora* recovered from overwintered soil.

Time	Cell number per gram soil				
	Replicate				Mean
Weeks	A	B	C	D	
0	9.0×10^{7}[a]	1.3×10^{8}	3.5×10^{7}	8.5×10^{6}	8.5×10^{7}
2	5.4×10^{6}	1.3×10^{7}	5.5×10^{6}	1.3×10^{7}	9.0×10^{6}
4	4.8×10^{6}	3.3×10^{7}	3.9×10^{6}	3.3×10^{6}	1.1×10^{7}
6	4.1×10^{6}	1.3×10^{6}	2.0×10^{6}	2.9×10^{6}	2.6×10^{6}
12	4.0×10^{4}	1.1×10^{5}	2.0×10^{4}	2.0×10^{4}	4.8×10^{4}
25	NR[b]	NR	NR	NR	
27	$R^{E}(2.6 \times 10^{7})$[c]	$R^{E}(1.1 \times 10^{8})$	$R^{E}(6.9 \times 10^{7})$	$R^{E}(1.0 \times 10^{8})$	(7.5×10^{7})
29	$R^{E}(5.9 \times 10^{7})$	$R^{E}(6.9 \times 10^{7})$	$R^{E}(1.3 \times 10^{8})$	$R^{E}(8.9 \times 10^{7})$	(8.8×10^{7})
31	$R^{E}(5.6 \times 10^{7})$	$R^{E}(6.2 \times 10^{7})$	$R^{E}(4.4 \times 10^{7})$	$R^{E}(2.5 \times 10^{7})$	(4.7×10^{7})
33	$R^{E}(1.0 \times 10^{7})$	$R^{E}(1.4 \times 10^{7})$	$R^{E}(1.8 \times 10^{7})$	$R^{E}(4.0 \times 10^{7})$	(2.0×10^{7})

[a] Selective medium used; lower limit of recovery is 10^{2} cells per gram soil.

[b] Not recovered with selective medium.

[c] Recovered with enrichment culture technique; values in parenthesis do not represent actual numbers.

TABLE 7. C-2 isolate of Erwinia carotovora variety carotovora recovered from overwintered soil.

Time	Cell number per gram soil				
	Replicate				
Weeks	A	B	C	D	Mean
0	2.0×10^{7}[a]	2.7×10^{7}	2.1×10^{7}	1.4×10^{7}	2.1×10^{7}
2	8.0×10^{4}	5.0×10^{4}	1.0×10^{4}	4.0×10^{4}	4.5×10^{4}
4	1.9×10^{4}	8.5×10^{3}	1.2×10^{5}	4.0×10^{3}	3.8×10^{4}
6	9.0×10^{3}	8.0×10^{4}	2.0×10^{5}	5.5×10^{3}	7.2×10^{4}
12	1.0×10^{2}	8.5×10^{2}	1.1×10^{3}	6.7×10^{2}	6.8×10^{2}
25	NR[b]	NR	NR	NR	
27	$R^{E}(2.4 \times 10^{6})$[c]	$R^{E}(1.0 \times 10^{5})$	$R^{E}(1.1 \times 10^{6})$	$R^{E}(1.7 \times 10^{6})$	(1.3×10^{6})
29	$R^{E}(4.5 \times 10^{6})$	$R^{E}(7.9 \times 10^{6})$	$R^{E}(4.0 \times 10^{6})$	$R^{E}(7.5 \times 10^{6})$	(5.8×10^{6})
31	$R^{E}(1.9 \times 10^{6})$	$R^{E}(3.2 \times 10^{6})$	$R^{E}(2.1 \times 10^{6})$	$R^{E}(1.2 \times 10^{6})$	(2.1×10^{6})
33	$R^{E}(1.2 \times 10^{6})$	$R^{E}(4.3 \times 10^{6})$	$R^{E}(3.5 \times 10^{6})$	$R^{E}(1.0 \times 10^{6})$	(2.5×10^{6})

[a] Selective medium used; lower limit of recovery is 10^{2} cells per gram soil.

[b] Not recovered with selective medium.

[c] Recovered with enrichment culture technique; values in parenthesis do not represent actual numbers.

TABLE 8. A-1 isolate of Erwinia carotovora variety atroseptica recovered from overwintered soil.

Time	Cell number per gram soil				
	Replicate				
Weeks	A	B	C	D	Mean
0	3.0×10^{6}[a]	4.0×10^{6}	1.4×10^{7}	4.0×10^{6}	6.1×10^{6}
2	6.0×10^{5}	1.4×10^{5}	2.5×10^{5}	1.4×10^{5}	2.8×10^{5}
4	3.0×10^{4}	1.6×10^{5}	8.5×10^{4}	3.0×10^{4}	7.6×10^{4}
6	5.0×10^{2}	7.0×10^{2}	7.0×10^{2}	6.2×10^{2}	6.3×10^{2}
12	NR[b]	NR	NR	NR	
25	NR[Ec]	NR^{E}	NR^{E}	NR^{E}	
27	NR^{E}	NR^{E}	NR^{E}	NR^{E}	
29	NR^{E}	NR^{E}	NR^{E}	NR^{E}	
31	NR^{E}	NR^{E}	NR^{E}	NR^{E}	
33	NR^{E}	NR^{E}	NR^{E}	NR^{E}	

[a] Selective medium used; lower limit of recovery is 10^{2} cells per gram soil.

[b] Not recovered with selective medium.

[c] Not recovered with enrichment culture technique.

TABLE 9. A-2 isolate of Erwinia carotovora variety atroseptica recovered from overwintered soil.

Time	Cell number per gram soil				
	Replicate				
Weeks	A	B	C	D	Mean
0	1.0×10^{6}[a]	2.0×10^{6}	2.0×10^{6}	1.0×10^{6}	1.5×10^{6}
2	1.1×10^{5}	9.0×10^{4}	1.5×10^{5}	8.5×10^{4}	1.1×10^{5}
4	2.0×10^{4}	1.3×10^{4}	1.5×10^{4}	2.0×10^{4}	1.7×10^{4}
6	1.0×10^{3}	1.0×10^{3}	1.0×10^{4}	1.6×10^{4}	7.1×10^{3}
12	NR[b]	NR	NR	NR	
25	NR^{Ec}	NR^{E}	NR^{E}	NR^{E}	
27	NR^{E}	NR^{E}	NR^{E}	NR^{E}	
29	NR^{E}	NR^{E}	NR^{E}	NR^{E}	
31	NR^{E}	NR^{E}	NR^{E}	NR^{E}	
33	NR^{E}	NR^{E}	NR^{E}	NR^{E}	

[a] Selective medium used; lower limit of recovery is 10^{2} cells per gram soil.

[b] Not recovered with selective medium.

[c] Not recovered with enrichment culture technique.