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Changes in microbial populations on fresh cut spinach

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Abstract

The microbial populations found on fresh-cut spinach leaves that were stored in gas permeable bags at 10°C for 12 days were examined and identified. The microorganisms consisted of mesophilic aerobic bacteria, psychrotrophic bacteria, Pseudomonadaceae, Enterobacteriaceae, Micrococcaceae, lactic acid bacteria and yeasts. Populations of mesophiles, psychrotrophs, Pseudomonadaceae and Enterobacteriaceae increased sharply during the storage period. The initial populations were 10^7, 10^6, 10^6 and 10^4 CFU.g^{-1} respectively. Populations reached 10^{10} for the mesophiles, psychrotrophs and Pseudomonadaceae and 10^7 CFU.g^{-1} for Enterobacteriaceae after 12 days of storage. Micrococcaceae, lactic acid bacteria and yeasts remained constant (10^3–10^4 CFU.g^{-1}). The majority of the bacterial isolates were identified as *Pseudomonas fluorescens*, *Aeromonas caviae* and *Staphylococcus xylosus*. The yeasts, which were most frequently isolated, were classified in the genus *Cryptococcus*. No pathogens such as *Listeria monocytogenes* and *Salmonella* were detected. Observations with low temperature scanning electron microscopy (LTSEM) indicated that the microorganisms were not present on the surface of healthy unbroken leaves. Alternatively, they were found in areas where the cuticle was broken and could be seen infecting the internal palisade parenchyma.

Keywords: Spinach; Microorganisms; Low temperature scanning electron microscopy

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1. Introduction

Fresh-cut vegetables are very popular because of their convenience, but they provide an ideal medium for the growth of various microorganisms (Brackett, 1987; Magnuson et al., 1990; Nguyen-the and Carlin, 1994). These products require refrigerated storage but the low temperature does not prevent the development of psychrotrophic microorganisms, particularly saprophytic fluorescent pseudomonads (Witter, 1961; Ingram, 1965) which are responsible for spoilage, as well as a few pathogens, such as *Listeria monocytogenes*, and *Aeromonas hydrophila* (Palumbo, 1986; Doyle, 1990; Brackett, 1992a). Reports also indicate that fresh-cut vegetables may be occasionally contaminated with foodborne pathogenic bacteria such as *L. monocytogenes* (Beuchat et al., 1987; Sizmur and Walker, 1988) and *Salmonella* spp. (O'Mahony et al., 1990). Although most fresh-cut vegetables are less contaminated than intact vegetables as they are washed with chlorinated water, slicing and shredding procedures as well as improper refrigeration during storage have been associated with an increase in the number of mesophilic aerobic microorganisms (Brackett, 1992a; Nguyen-the and Carlin, 1994). Therefore, concern exists about how microorganisms affect the quality and the safety of fresh-cut vegetables.

Fresh-cut spinach have received little attention in terms of microbiological considerations. Consequently, a study was undertaken to identify and quantify Pseudomonadaceae, Enterobacteriaceae, fecal coliforms, Micrococcaceae, lactic acid bacteria and yeasts that may be present on fresh-cut spinach leaves. Particular attention was given to the pathogens *Monocytogenes* and *Salmonella*. The changes in the mesophilic and psychrotrophic aerobic microorganisms and specific microbial populations were also followed during storage of the product. A scanning electron microscope (SEM) was used to examine the location of invasion of the microorganisms on spinach leaves. A variation of this technique, referred to as low temperature scanning electron microscopy (LTSEM) allowed us to observe the spinach leaves and the associated microorganisms in their frozen-hydrated condition. This procedure avoided artifacts such as mechanical damage, extraction, and shrinkage, that are associated with chemical processing, dehydration and critical point drying-conventional procedures used for SEM preparation (Wergin and Erbe, 1989; Wergin and Erbe, 1992).

2. Materials and methods

2.1. Spinach

Spinach leaves (*Spinacia oleracea* L., variety New Jersey) were obtained from a local fresh-cut produce processor and stored in gas permeable bags at 10°C for 12 days. Transmission rates of O_2 and CO_2 were 31 and 124 $ml \cdot m^{-2} \cdot day^{-1} \cdot atm^{-1}$ at 23°C, respectively. Three bags were analysed after 0, 2, 5, 8 and 12 days of storage.

2.2. Texture

Texture was measured as the force required to shear 20 g of spinach leaves using a Food Technology Corporation Texture Test System[1] (Model TMS-90) equipped with a standard shear-compression cell (Model CS-1). Spinach leaves were placed in the cell box perpendicular to the 10 shear blades. The force was expressed in Newtons (N).

2.3. External and total pH

2.3.1. Total pH

A 20 g sample of spinach leaves in 40 ml of deionized water was blended for 1 min and the pH of the macerate was measured with an Orion Research pH meter (Model 811).

2.3.2. External pH

Surface of spinach leaves was wiped with a piece of sampling sheet soaked in deionized water and pH was measured with a Cardy twin pH B-113.

2.4. Enumeration and differentiation of microorganisms

Spinach leaves (20 g) were macerated in 40 ml of sterile peptone water, pH 7.4, with a 400 Lab Stomacher (Seward Medical, London, UK). A sample of each homogenate or appropriate dilution was spread on agar plates using a Spiral Plate System (Autoplate Model 3000, Spiral Biotech, Bethesda, MD). The enumeration and differentiation of microorganisms and particular microbial groups or species were performed by using the following culture media and culture conditions: (1) tryptic soy agar (TSA, Difco Laboratories, Detroit, MI) incubated at 30°C for 24 h for total mesophilic aerobic microorganisms or incubated at 5°C for 4 days for psychrotrophic aerobic microorganisms (Brackett, 1989); (2) *Pseudomonas* selective isolation agar (PSIA) prepared according to Krueger and Sheik (1987) and incubated at 30°C for 24 h for pseudomonads; (3) Violet red bile glucose agar (VRBG, Difco) incubated at 37°C for 24 h for total Enterobacteriaceae except *Erwinia* (Guiraud and Galzy, 1980); (4) Violet red bile agar (VRBA, Difco) incubated at 44°C for 24 h for coliforms (Guiraud and Galzy, 1980); (5) Chapman agar medium incubated at 30°C for 24–48 h for Micrococcaceae (Guiraud and Galzy, 1980); (6) MRS agar medium with the addition of bromocresol green (100 ppm) incubated under anaerobic conditions at 30°C for 3 days for lactic acid bacteria (Carlin et al., 1989); (7) Desoxycholate citrate lactose agar (DCL) incubated at 37°C for 24 h for *Salmonella* (Guiraud and Galzy, 1980); (8) Potato dextrose agar (PDA, Difco) with addition of chloramphenicol (500 ppm) incubated at 30°C for 36 h (Babic et al., 1992) for yeasts and; (9) Paton's medium (Paton, 1959), nutrient gelatin (Sigma)

[1] Use of a company or product name by the USDA does not imply approval or recommendation of the product to the exclusion of others which also may be suitable.

and lipase method (Joffin and Leyral, 1991) incubated at 25°C for 3 days for pectolytic, proteolytic and lipolytic bacteria, respectively. The detection of *Listeria* spp. in spinach samples was performed according to Fraser and Sperber (1988). Portions (1 ml) of spinach macerate were enriched in 9 ml of Fraser broth (Fraser Broth Base used with Bacto Fraser Broth Supplement, Difco) for 24 h at 30°C. Zero-point-one millilitres of this primary enrichment was added to 10 ml of Fraser broth and this secondary enrichment was incubated at 30°C for 24 h. Cultures were then streaked onto Oxford medium (Oxford Medium Base used with Bacto Oxford Antimicrobic Supplement, Difco) and plates were incubated at 30°C for 36 h.

2.5. Isolation and identification of microorganisms

Isolation of single colonies was performed by selecting randomly the square root of the total number of colonies counted on each plate. Identification was conducted according to Lelliot et al. (1966); Lodder (1970); Guiraud and Galzy (1980); Kreger-van-Rij, 1984 and Bergey's manual (Krieg and Holt, 1984; Sneath et al., 1986). All isolated strains were purified and classified according to their cellular morphology observed by phase contrast microscopy ($\times$ 1000), their biochemical, physiological and sexual characteristics using the Gram enzymatical test (Cerny, 1976), chemical tests, such as oxidase and catalase and growth on King medium B (Difco) to reveal the fluorescence of pseudomonads.

Gram-negative, oxidase-positive and fluorescent bacteria were classified as described by Lelliot et al. (1966). They were further tested for arginine dihydrolase, gelatinase, nitrate reductase (except that liquid medium was used instead of agar medium) and levan production. Gram-negative non-fluorescent bacteria isolated from PSIA medium and Enterobacteriaceae were identified using API 20E strips (Biomerieux Vitek, Inc., Hazelwood, MO) and the Biolog's Automated System (Biolog, Inc., Hayward, CA). Strains characterized as suspect colorless colonies on DCL medium were tested for *Salmonella* reactions according to a procedure obtained from the Food Microbiology Laboratory of the Food Safety Inspection Service (USDA, Beltsville, MD). Strains were inoculated on Triple Sugar Iron Agar (TSIA, Difco) and Lysine Iron Agar (LIA, Difco). The inoculation was performed in tandem by stabbing the butts and streaking the slants in one operation. The cultures were incubated at 35°C for 24 h. The strain *Salmonella enteriditis* stk 1007 was used as control. Media were examined for (1) yellow butts and red slants on TSIA, and (2) purple butts with a blackening and production of gas on LIA, which are typical of *Salmonella* reactions. Gram-positive bacteria isolated from Chapman agar medium were identified with the API STAPH (Biomerieux Vitek, Inc., Hazelwood, MO). The identification of yeasts was conducted as follows: cell morphology was observed after growth in YG liquid medium (yeast extract 5 $g \cdot l^{-1}$, glucose 5 $g \cdot l^{-1}$), formation of pseudo or true mycelium was tested on Corn Meal Agar (Difco) with the slide culture procedure (Kreger-van-Rij, 1984), the assimilation of twenty carbohydrates was tested with API 20C strips (Biomerieux Vitek, Inc., Hazelwood, MO), the assimilation of nitrates was tested in Wickerham Yeast Carbon Base medium (YCB, Difco) and the fermentation of glucose in

Wickerham medium (Guiraud and Galzy, 1980). Cultures were incubated at 30°C for 2–3 days. The frequency of species was calculated as the number of isolates of a species over the total number of isolates.

2.6. Low temperature scanning electron microscopy (LTSEM)

A Hitachi S-4100 field emission low temperature scanning electron microscope (Hitachi Scientific Instruments, Mountain View, CA) equipped with an Oxford CT 1500HF Cryotrans System (Oxford Instruments, Eynsham, UK) was used to examine spinach leaves that were stored for 12 days. One-cm^2 segments of the leaf showing either broken necrotic areas or no signs of injury were positioned vertically or flat onto a specimen holder and mounted with a cryo-adhesive (Tissue Tek, Miles Scientific, Naperville, IL). The holders were immediately plunge-frozen in liquid nitrogen and then transferred under vacuum to the cold stage in the pre-chamber of the cryosystem. To examine the internal tissue, the vertically mounted frozen samples were fractured with a pre-cooled blade through either a necrotic area from an infected region of the leaf or a normal area from a healthy region. The fractured samples were etched for 8 min in the pre-chamber by raising the temperature of the cold stage to −90°C, sputter-coated with Pt for 2 min and then transferred to the cryostage of the LTSEM for observation. All samples were observed with an accelerating voltage of 10 kV and images were recorded onto a Polaroid type 55P/N film.

3. Results

3.1. Changes in texture and pH of spinach leaves during storage

The texture of spinach leaves decreased sharply after the second day of storage (Table 1). The external pH, which was slightly acidic, remained relatively stable during the first 8 days of storage and then rose sharply. Total pH, which was more neutral, increased gradually during storage.

Table 1
Changes in texture, plus external and total pH of fresh-cut spinach during storage in gas permeable bags at 10°C

Days of storage	Texture (N)	External pH	Total pH
0	1722 ± 80	5.0 ± 0.20	6.4 ± 0.10
2	1822 ± 85	5.1 ± 0.06	6.4 ± 0.00
5	1519 ± 120	5.2 ± 0.06	6.8 ± 0.15
8	1371 ± 126	5.6 ± 0.50	7.3 ± 0.20
12	1324 ± 150	8.7 ± 0.20	7.3 ± 0.10

Averages and S.D.s were calculated for 3 replicates

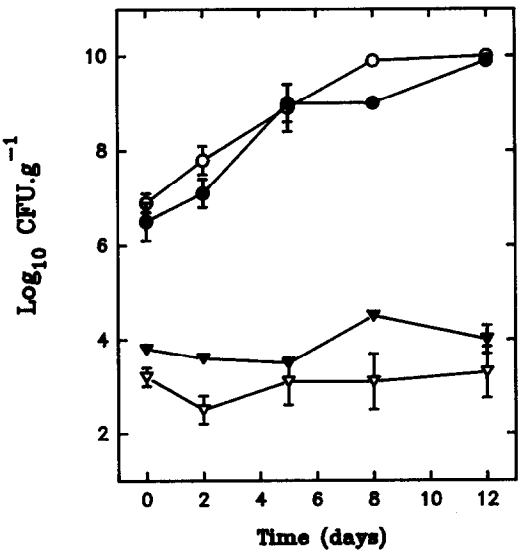

Fig. 1. Changes in microbial populations, (●) mesophilic aerobic microorganisms, (○) psychrotrophic aerobic microorganisms, (▼) yeasts and (▽) lactic acid bacteria on fresh-cut spinach stored in gas permeable bags at 10°C. Vertical lines represent the S.D. on 3 replicates.

3.2. Microbial changes during storage

Figs. 1 and 2 show the changes in the microbial populations on fresh-cut spinach leaves stored in gas-permeable bags at 10°C. Total populations of mesophilic and psychrotrophic aerobic microflora, initially 10^6–10^7 CFU/g, increased sharply during the first 8 days of storage and then remained relatively constant at 10^{10} CFU/g. Pseudomonadaceae, which were the predominant group of bacteria that were found 10^2–10^3 times greater than others, showed growth similar to the mesophiles and psychrotrophs. Populations of lactic acid bacteria, yeasts and Micrococcaceae remained at lower levels of 10^3–10^4 CFU/g and 10^2 CFU/g, respectively. The population of total Enterobacteriaceae, grown at 37°C, increased from 5 x 10^4 CFU/g at the initial day to 10^7 CFU/g by the end of the storage period. Coliforms, capable of growth at 44°C, were detected only after 12 days of storage at 5 × 10^3 CFU/g.

3.3. Type and frequency of microorganisms

The different species of bacteria and yeasts are listed in Table 2. Most of the bacterial isolates were characterized as Gram-negative short rods, oxidase, fluorescence and arginine positive and belonging to the genus *Pseudomonas*. The main species were identified as *Pseudomonas fluorescens* of different biovars. Isolates which were characterized as nitrate reductase positive, levan negative and no growth at 41°C (Sneath et al., 1986) were identified as biovar III. A second group

of isolates, which did not reduce nitrates but produced levan, was identified as *fluorescens aureofasciens* or *fluorescens* biovar I, and those which did not produce levan but hydrolysed gelatin were identified as biovar V. The biovar III represented 75% of the Pseudomonads by the end of the storage.

A high percentage (80%) of *Pseudomonas* isolates had pectolytic, proteolytic and lipolytic activities. Among the Enterobacteriaceae, *Citrobacter freundii* and *Serratia liquefasciens* or *marescens* were the only species identified with a good percentage of probability (60–80%). The identity of the remaining isolates was uncertain. From the 25 strains isolated from DCL medium, none gave a typical *Salmonella* reaction on both PSIA and LIA media. Half of these isolates were identified as *Aeromonas caviae* during the entire storage period. Coliforms, which were detected only on the 12th day of storage, were represented solely by the species *Klebsiella pneumoniae*. Gram-positive bacteria could be separated into two groups, lactic acid bacteria which were not identified, and Micrococcaceae represented by only one species, *Staphylococcus xylosus* 2. This study also focused on the isolation of the foodborne pathogen *Listeria monocytogenes*. After enrichment, no *Listeria* spp. were detected in any of the samples tested.

Several species of yeasts were also isolated from fresh-cut spinach leaves. A large group of isolates formed white colonies on agar medium, and ovoid cells with bipolar buddings in liquid medium but did not form pseudo-mycelium on slide culture. They did not ferment glucose, but assimilated inositol, maltose, sucrose and nitrates and therefore were identified as *Cryptococcus albidus*. Isolates that assimilated lactose were classified in the variety *albidus* and lactose- negative isolates were

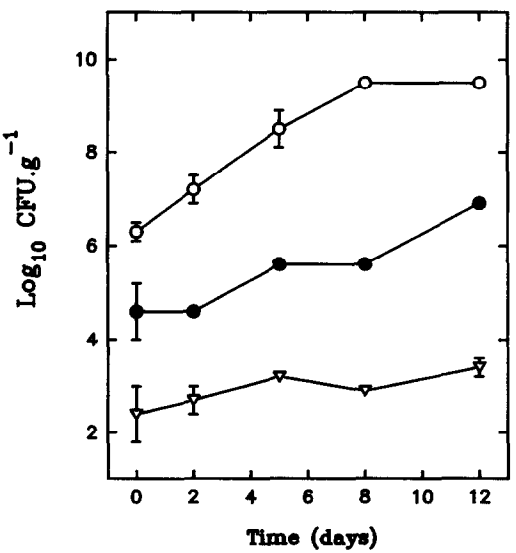

Fig. 2. Changes in microbial populations, (○) Pseudomonadaceae, (●) Enterobacteriaceae, (▽) Micrococcaceae on fresh-cut spinach stored in gas permeable bags at 10°C. Vertical lines represent the S.D. on 3 replicates.

Table 2
Number of bacteria and yeasts isolated from fresh-cut spinach stored in gas-permeable bags at 10°C

Group	Species	0 days	12 days
Pseudomonadaceae	Total no. of isolates	30	30
	Ps. fluorescens biov. III	8	23
	Ps. fluorescens biov. I or *aureofaciens*	15	0
	Ps. fluorescens biov. V	0	7
	Ps. putida	7	0
Enterobacteriaceae	Total no. of isolates	25	25
	S. liquefaciens or *marcescens*	6	8
	Citrobacter freundii	4	0
	Non-identified	15	17
Vibrionaceae	Total no. of isolates	25	25
	Aeromonas caviae	15	12
	Non-identified	10	13
Coliforms	*Klebsiella pneumoniae*	0	20
Micrococcaceae	*Staphylococcus xylosus* 2	15	15
Yeasts	Total of isolates	20	25
	Cr. albidus var. *diffluens*	15	8
	Cr. albidus var. *albidus*	0	5
	Cr. albidus	0	8
	Rhodotorula rubra	5	0

classified in the variety *diffluens*. Isolates that did not assimilate nitrates were identified as *Cryptococcus laurentii*. Some strains with orange colonies on agar medium that did not ferment glucose and did not assimilate inositol were identified as *Rhodotorula rubra*. The species *Cryptococcus albidus* was predominant, representing 75% of the total yeast population at day 0 and 92% on day 12.

3.4. Observation of spinach tissue and microorganisms by LTSEM

LTSEM was used to examine tissues that appeared undamaged and those which exhibited broken and necrotic areas. Freeze-fractures of the vertically mounted samples provided cross sections of the leaf in which the upper epidermis, palisade parenchyma, spongy parenchyma and lower epidermis could be distinguished (Fig. 3). The tissue segments that appeared normal at the time of sampling were free of microorganisms. Conversely, in the segments that had exhibited necrotic areas, the upper epidermis and the palisade parenchyma were covered with a continuous layer of bacteria (Fig. 4).

Observation of the surface of the leaf revealed the tops of the rounded epidermal cells and randomly distributed stomata. No microorganisms could be seen around the stomata. The cuticle was disrupted in the necrotic areas and the internal cells of

the palisade parenchyma were coated with bacteria that appeared to be associated with a film possibly consisting of glycocalyx (Fig. 5). The palisade parenchyma cells were separated one from one another by intercellular spaces that were filled with bacterial colonies (Fig. 6). Most of the bacterial cells consisted of short rods usually grouped in chains embedded in a meshwork that probably consisted of glycocalyx. This meshwork consisted of layers of filaments on which the bacteria developed (Fig. 7). The meshwork seemed to interconnect the cells in the palisade parenchyma thereby allowing the bacterial colonies to expand. Recent studies have shown that mucoid strains of saprophytic *P. fluorescens*, particularly biovars III and V as found in spinach leaves, produced exopolysaccharides involved in the attachment of bacteria to the host plant (Rainey, 1991; Fett et al., 1995) which protected them from adverse environmental conditions such as desiccation (Osphir and Gutnick, 1994).

4. Discussion

This study showed that fresh-cut spinach leaves contain high populations of mesophilic and psychrotrophic microorganisms, with *P. fluorescens* being the

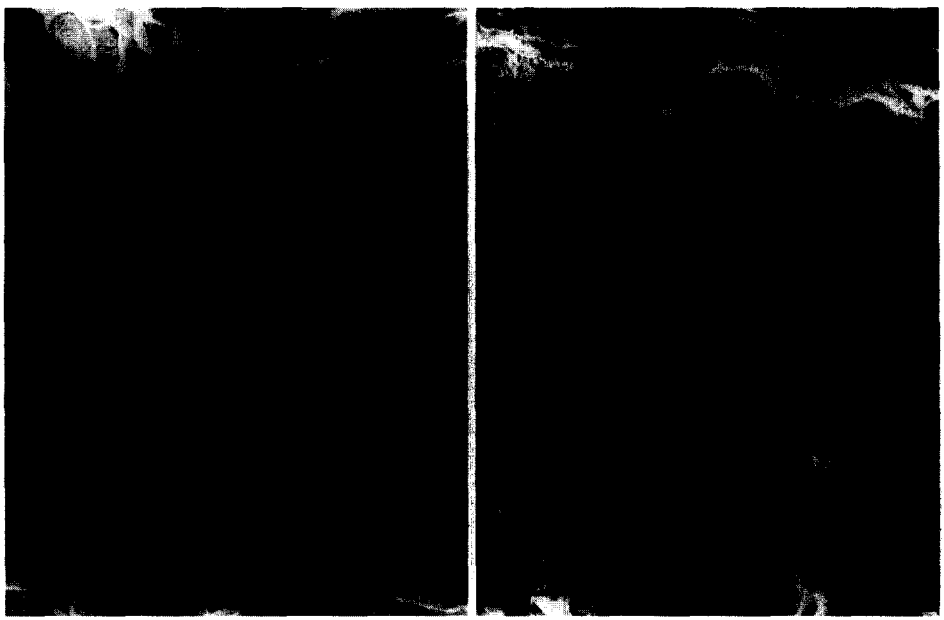

Fig. 3. Freeze-fractured image of a spinach leaf. The cross section of this healthy leaf segment revealed the: upper epidermis (ue) covered with the cuticle (cu), palisade parenchyma (p), spongy parenchyma (s) and lower epidermis (le). × 300. Bar = 60 μm.

Fig. 4. Spinach leaf freeze-fractured along a necrotic area. The palisade parenchyma is covered with a layer of bacteria (b). × 300. Bar = 70 μm.

Fig. 5. Upper surface of the leaf illustrating the cuticle (cu) with stomata (st). The necrotic areas, which are characterized by the separation or rupture of the protecting cuticle, reveals the cells (c) of the palisade parenchyma that are covered with a layer of bacteria (b). × 420. Bar = 50 μm.

Fig. 6. Palisade parenchyma cells covered with bacteria (b). Bacteria form a meshwork that engulfs the cells (c). Close associations can be seen between bacteria and the wall of the parenchyma cells (arrows). × 1350. Bar = 14 μm.

Fig. 7. Detail of a bacterium (b) embedded in glycocalyx (g). The glycocalyx forms a meshwork that interconnects the different cells of the palisade parenchyma (c). × 21 800. Bar = 1 μm.

predominant species. Mesophilic and psychrotrophic microbial populations were comparable throughout the storage. This observation is consistent with that of Garg et al. (1990) who reported similar data. The reason for this observation may be that many *Pseudomonas* species are psychrotrophs and according to Brackett (1989), a neutral rather than acidic pH, such as that found in spinach leaves might provide more favorable growth conditions for the psychrotrophs.

All of the *Pseudomonas* isolates were pectolytic; therefore they were probably involved in degradation of the textural quality of the spinach leaves. Fluorescent pectolytic pseudomonads have frequently been found to be involved in the deterioration of vegetables (Brocklehurst and Lund, 1981; Nguyen-the and Carlin, 1994). However, factors other than pectolytic enzymes might also be important in spoilage. Several investigators have recently suggested that viscosin, a potent peptidolipid biosurfactant produced by pectolytic *P. fluorescens*, might facilitate bacterial infection and the spread of decay on broccoli florets (Hildebrand, 1989; Laycock et al., 1991). The foodborne pathologic bacteria *Salmonella* spp. and *Listeria monocytogenes* were not detected. In contrast, Garcia-Villanova Ruiz et al. (1987) reported the presence of *Salmonella* on fresh spinach leaves. Coliforms, such as *Klebsiella pneumoniae* were only detected after 12 days of storage. *Klebsiella* may be responsible for food poisoning when encountered in high numbers in fresh fruits and vegetables (Guiraud and Galzy, 1980; Brackett, 1987; Nguyen-the and Carlin, 1994). Lactic acid bacteria, yeasts and Micrococcaceae did not appear to play a significant role in altering the structure of spinach leaves because their numbers remained relatively constant during storage. However, lactic acid bacteria have been identified as a cause of deterioration in several fresh-cut vegetables, particularly sliced and shredded carrots (Buick and Damouglou, 1987; Carlin et al., 1989) and yeasts have been responsible for the spoilage of various fruit and vegetable commodities (Guerzoni and Marchetti, 1987). Contamination of fresh-cut spinach may be explained by poor sanitation during processing. For example, Garg et al. (1990) and Brackett (1992b) reported the limitations of chlorine disinfection to eliminate intrinsic microorganisms, particularly Enterobacteriaceae.

LTSEM allowed us (1) to observe the specific areas where the bacterial infection occurred and (2) to determine the mode of infection of the bacteria within the palisade parenchyma. The bacteria did not appear to penetrate the leaf through stomata but rather seemed to concentrate in areas where the cuticle and underlying tissues had been physically damaged. This observation is consistent with that of Brocklehurst and Lund (1981) who reported that a wide range of pectolytic fluorescent pseudomonads isolated from celery petioles and cabbage leaves caused soft rot of wounded, but not unwounded tissues. In some conditions, the species *Pseudomonas marginalis* was reported to enter host plants through stomata (Dowson, 1941) but only after plant tissues were initially damaged (Friedman, 1951). In the palisade parenchyma tissue, bacteria that have pectolytic activity hydrolysed the middle lamellae which bind adjacent cells. This process of cell separation resulted in loss of tissue firmness and was consistent with our results of texture measurements. The bacteria appeared to be embedded in a glycocalyx that formed a bridging meshwork. This glycocalyx, which apparently allowed the bacteria to colonize

adjacent cells and provided a support and nutriment to the colony, may result from a combination of bacterial secretion and hydrolysis of the cell wall components.

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