

In vitro Inhibition of Soft-Rotting Bacteria by EDTA and Nisin and in vivo Response on Inoculated Fresh Cut Carrots

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ABSTRACT

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EDTA and the antibiotic nisin, in combination with heat, were tested for inhibition of growth of six pectolytic, soft-rotting bacteria in 80% trypticase soy broth (TSB). Fifty percent reduction of growth by EDTA at 25°C in TSB occurred at 3.24 mM for *Erwinia chrysanthemi*, 2.57 mM for *Pseudomonas fluorescens*, 0.96 mM for *E. carotovora* (subsp. *carotovora*), 0.48 mM for *P. viridiflava*, 0.17 mM for *Xanthomonas campestris* (pv. *campestris*), and 0.16 mM for *Cytophaga johnsonae*. Nisin at 50 µg/ml was effective against *X. campestris* and *C. johnsonae* (over 90% inhibition of growth) but not against the other four bacteria (less than 20% inhibition), which are the more economically important soft-rotters. Combinations of EDTA and nisin were synergistic. A combination of 0.3 mM EDTA + nisin at 50 µg/ml inhibited growth of *E. carotovora*, *E. chrysanthemi*, and *P. viridiflava* by over 70%, and growth of *P. fluorescens* by 37%. Hot water treatments for 0.3 min at 37 or 49°C reduced survival of bacteria in the presence of EDTA + nisin, but not of EDTA, nisin, or water alone. EDTA + nisin at 37°C reduced CFU/ml of *E. carotovora*, *E. chrysanthemi*, *P. fluorescens*, and *P. viridiflava* by 2 log units, and at 49°C by 3 log units, compared with the 25°C treatment. Decay of carrot disks inoculated at two inoculum levels (10^3 and 10^4 CFU per disk) with *E. carotovora*, *P. fluorescens*, or *P. viridiflava* was reduced by a 1.5-min immersion in 45°C water, with or without EDTA and nisin additives. Immersion in 0.3 mM EDTA + nisin at 15 to 50 µg/ml at 45°C reduced decay due to *E. carotovora* and to *P. fluorescens* at the lower inoculum level by an average of about 50% compared with water alone at 45°C, but differences were statistically significant only at the 90% level of confidence and no different than a standard chlorine dip, current commercial practice for cut carrot slices.

Additional keywords: bacterial soft rot

Salts of EDTA have long been used as antimicrobial agents, particularly against bacteria (6,19). They have also been effective as enhancers of other antimicrobial agents, such as lysozyme, antibiotics, and irradiation, by increasing permeability of bacterial membranes or by removal or destruction of covalently bound lipid components (3,7,12,13,20). Heat has also been used as an antimicrobial agent and to increase effectiveness of chemical treatments (4).

Zucker and Hankin (21) reported a reduction of tissue maceration in potato disks inoculated with the soft-rotting bacteria *Erwinia carotovora* and *Pseudomonas marginalis* (i.e., pectolytic *P. fluorescens*) by 5 mM and 10 mM tetrasodium EDTA,

respectively. The mode of action was attributed to inhibition of the bacterial enzyme pectate lyase (15). In vitro survival of these bacteria was reduced approximately 99% by a 1-h exposure to 0.75 mM EDTA.

Bacterial soft rot, the leading biological cause of postharvest losses of fruits and vegetables, is caused by a diverse group of pectolytic plant pathogens, which includes, in addition to *E. carotovora* and *P. fluorescens*, *Pseudomonas viridiflava*, *Xanthomonas campestris*, *Cytophaga johnsonae*, and *Erwinia chrysanthemi* (8–11). The most effective control of this disease complex, other than by reducing injury to the commodity during harvesting and marketing, is by sanitation, refrigeration, and surface-sterilization with chlorine (11).

The commercial popularity of fresh-cut produce, such as carrot and celery slices and prepared salads, suggested an opportunity to evaluate the effectiveness of EDTA in combination with an antibiotic to reduce bacterial soft rot. Nisin is a bacteriocin of the lantibiotic group with broad activity, and thus it is similar to antibiotics. Nisin was recently approved in the United States for food use (5). Although most effective against gram-positive bacteria, its range

includes some gram-negative bacteria (3,16,17). Nisin has not been tested against the plant pathogenic soft-rotting bacteria, nor is there published information on the effect of EDTA or nisin on fresh-cut produce such as carrots. This report is an evaluation of the effectiveness of EDTA and nisin for controlling gram-negative soft-rotting bacteria in vitro and in vivo on cut carrots.

MATERIALS AND METHODS

Bacteria. One strain each of six gram-negative soft-rotting bacteria was used for this study: *E. carotovora* var. *carotovora* 22 (H. Moline, USDA, Beltsville, MD), *E. chrysanthemi* 16 (A. Chatterjee, University of Missouri, Columbia, MO), *P. fluorescens* 8-7 isolated from soft-rotted bell peppers (this laboratory), *P. viridiflava* SF312 isolated from soft-rotted squash (8), *X. campestris* 1 (E. Civerolo, USDA, Beltsville, MD), and *C. johnsonae* W101 isolated from soft-rotted watermelon (9,10). Bacteria were stored in trypticase soy broth (TSB; Difco Laboratories, Detroit, MI) containing 5% dimethyl sulfoxide at -90°C, and maintained on *Pseudomonas* Agar F (PAF, Difco) at 25°C for growth studies and for inoculation of plant tissues.

In vitro growth and inhibition tests. Bacteria were grown aerobically at 25°C with agitation in 80% TSB for general growth studies and on 50% pectin-MY broth medium to test pectate lyase (PL) inhibition (8). Culture tubes (16 × 125 mm) containing 6.3 ml of media were inoculated with 0.7 ml of 16-h late log phase cells (grown on TSB and twice washed) at three different inoculum levels: 10^5 , 10^6 , and 10^7 CFU/ml. Growth rates were measured by turbidometry at an optical density of 590 nm at 2- to 4-h intervals during the first 24 h and intermittently thereafter up to 54 h, with uninoculated media used as blanks (1).

In inhibition studies, the disodium salt of EDTA (Sigma, St. Louis, MO) was added to the media at 0, 0.01, 0.1, 1, and 10 mM final concentration. Nisin (Sigma), tested in 80% TSB medium, was added at 5, 15, 50, and 150 µg/ml final concentration, with or without EDTA at 0.3 mM, and growth was measured by turbidometry. Percent inhibition was calculated by comparing optical densities of treated and untreated control cultures when the controls were at the end of log phase growth. Log phase for the controls with the highest

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starting inoculum usually ended between 8 and 28 h, depending on the bacterium, and between 28 and 54 h for those with the lowest starting inoculum. The EDTA-nisin combination treatments were additionally tested at 25, 37, and 49°C on 80% TSB for 0, 0.3, 1, 3, and 10 min exposure times. Survival was determined by dilutions of unwashed cells in sterile distilled water (18). In order to conserve volume, 20- μ l

aliquots were withdrawn by micropipette and serially diluted 2 to 6 logs with 180 μ l of water. Ten μ l of diluents were then streaked on PAF medium in 60-mm plates and incubated for 24 to 48 h at 25°C. Correction factors were applied to convert colony counts to CFU/ml. Controls were included to empirically establish that any residual EDTA or nisin carried by the dilutions had no effect on CFU determinations.

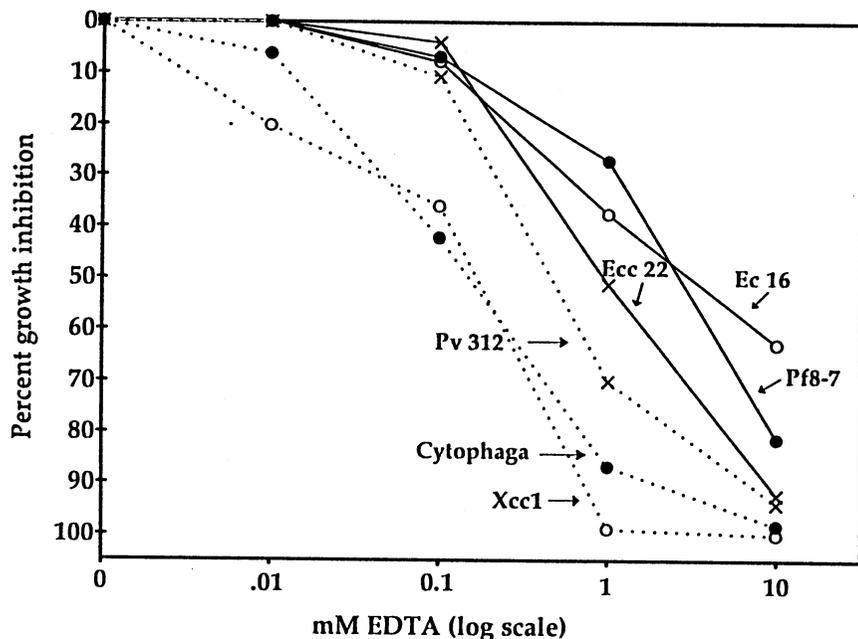


Fig. 1. Percent inhibition of growth at 25°C of six soft-rotting bacteria by EDTA in 80% trypticase soy broth. Each point represents an average of three tests, each with a different concentration of inoculum (10^5 , 10^6 , and 10^7 CFU/ml), replicated three times. Ecc22 = *Erwinia carotovora* var. *carotovora* 22, Ec16 = *E. chrysanthemi* 16, Pf8-7 = *Pseudomonas fluorescens* 8-7, Pv312 = *P. viridiflava* SF312, Xcc1 = *Xanthomonas campestris* 1, and Cytophaga = *C. johnsonae* W101.

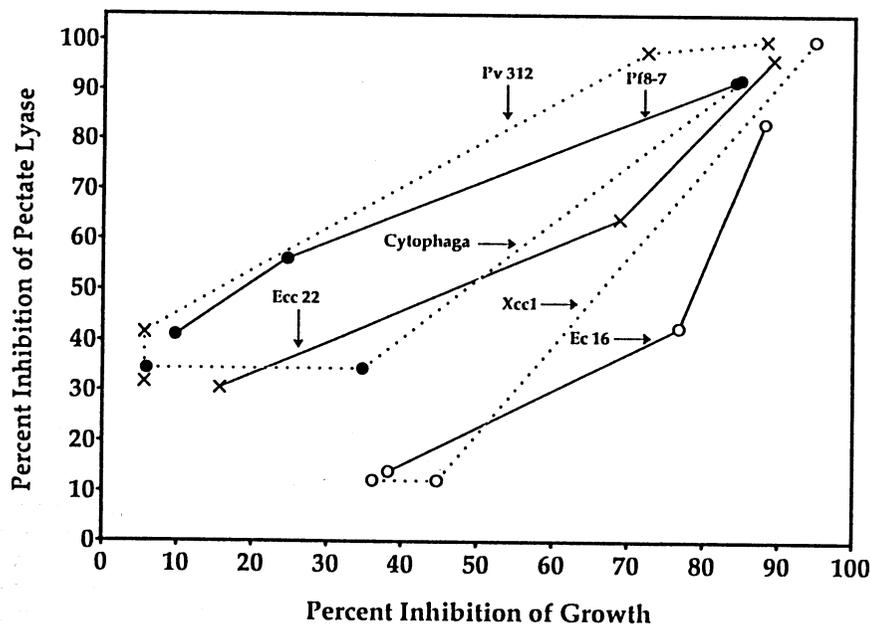


Fig. 2. Relationship between inhibition of growth and reduction of pectate lyase activity for six soft-rotting bacteria growing in 80% trypticase soy broth at 25°C. Each point represents the average of three different inoculum concentrations of bacteria, replicated three times. Ecc22 = *Erwinia carotovora* var. *carotovora* 22, Ec16 = *E. chrysanthemi* 16, Pf8-7 = *Pseudomonas fluorescens* 8-7, Pv312 = *P. viridiflava* SF312, Xcc1 = *Xanthomonas campestris* 1, and Cytophaga = *C. johnsonae* W101.

Production of the bacterial enzyme pectate lyase (PL) was measured for cells grown on pectin-MY medium in the presence of EDTA in order to determine if enzyme activity was inhibited by EDTA to the same extent as growth. One-half ml of culture fluid was removed from each tube when controls had reached the end of log phase growth. PL activity was measured spectrophotometrically, and controls were included to ensure that trace levels of EDTA present in assay cuvettes did not affect the assay (8). Growth inhibition and enzyme tests were repeated three times.

Carrot disk assays. Mature and prepeeled baby carrots (*Daucus carota* L.) were obtained from local markets, surface sterilized with 0.5% sodium hypochlorite for 10 min, rinsed in sterile H_2O , and aseptically cross-sectioned in disks 2 to 3 mm thick. Preliminary tests were then conducted on phytotoxicity of heat, EDTA, and nisin alone and in combination. Slices were inoculated with 10 μ l of suspensions of *E. carotovora*, *P. fluorescens*, or *P. viridiflava* at 10^5 or 10^6 CFU/ml (10^3 and 10^4 CFU/disk). Inocula were grown 24 h on PAF, suspended in sterile distilled water, and placed as a single drop at the center of each slice, then incubated for 2 h at 25°C to allow establishment of the bacteria on the cut surfaces. Randomly selected groups of slices were then immersed in 500 ml of the solutions for 1.5 min at given concentrations and temperatures, with mild agitation. Treatments were the following: water alone at 25 or 45°C ($\pm 2^\circ C$); 0.3 mM EDTA with nisin at 0, 5, 15, 50, or 150 μ g/ml at 25 and 45°C ($\pm 2^\circ C$); chlorine (sodium hypochlorite at 100 μ g/ml, pH 6.7) at 25°C; and a dry control. The water treatment at 25°C was considered the wet control. Each treatment was performed with five to six carrot sections. The pH of the chlorine solution was checked after one series of tests to monitor and verify the stability of free chlorine under experimental conditions. Treated disks were placed in sterile petri dishes, incubated at 25°C, and scored for soft rot development at 24-h intervals for 3 consecutive days. Scores were based on lesion diameter: 1 = less than 2 mm, 2 = 2 to 10 mm, 3 = 10 to 20 mm, and 4 = over 20 mm. Experiments in which the wet controls developed no soft rot or were 100% rotted (average score of 4) were disregarded because of too little or too heavy infection. Successful tests were repeated 21 times with *P. viridiflava* and 6 times each with *E. carotovora* and *P. fluorescens*. Data for the array of tests were averaged and statistically analyzed by an analysis of variance and by Duncan's multiple range test as modified by Tukey (14).

RESULTS

In vitro inhibition. Soft rot bacteria differed in sensitivity to EDTA. Average 50% effective dose (ED_{50}) levels in 80% TSB

medium for the three levels of inoculum were 0.16 mM for *C. johnsonae*, 0.17 mM for *X. campestris*, 0.96 mM for *P. viridiflava*, 0.96 mM for *E. carotovora*, 2.57 mM for *P. fluorescens*, and 3.24 mM for *E. chrysanthemi* (Fig. 1). Initial population level was a significant factor in growth inhibition. Based on the average of the three levels of inoculum, 0.3 mM EDTA reduced growth of *X. campestris* by approximately 65%, *C. johnsonae* by 63%, *P. viridiflava* by 39%, *E. carotovora* by 27%, *E. chrysanthemi* by 22%, and *P. fluorescens* by 18%, for a combined average of 39%. Individual averages, however, for inoculum levels of 10^5 , 10^6 , and 10^7 CFU/ml were 18, 39, and 69%, respectively (data not shown).

The relationship between pectate lyase activity and EDTA-induced growth inhibition differed with each organism. Enzyme inhibition was generally proportional to growth inhibition in *E. carotovora* and *C. johnsonae*, where 50% inhibition of growth (an average of three inoculum levels) resulted in approximately 50% reduction in enzyme activity. With *X. campestris* and *E. chrysanthemi*, 50% growth inhibition resulted in approximately 24% reduction in enzyme activity. With *P. fluorescens* and *P. viridiflava*; however, 50% inhibition of growth rate resulted in over 70% reduction of enzyme activity (Fig. 2).

Nisin was highly toxic to *X. campestris* and *C. johnsonae*, with 5 μ g/ml inhibiting growth of each by over 60%. However, a dose of 150 mg/ml inhibited growth of *P. viridiflava* by 42%, and of *E. chrysanthemi* and *E. carotovora* by approximately 20%, while *P. fluorescens* was not signifi-

cantly affected (Fig. 3A). In the presence of 0.3 mM EDTA, however, nisin inhibited all bacteria except *P. fluorescens* by over 50% at 5 μ g/ml, by at least 70% at 50 μ g/ml, and by 80% or over at 150 μ g/ml (Fig. 3B). *P. fluorescens* was least affected by the combination treatment, with a 37% reduction of growth by nisin at 50 μ g/ml + EDTA and a 60% reduction by nisin at 150 μ g/ml + EDTA.

Heat in combination with EDTA was an effective enhancer of antibiotic activity. Combining data for *E. carotovora*, *E. chrysanthemi*, *P. viridiflava*, and *P. fluorescens*, 0.3-min treatments with nisin at 50 μ g/ml + 0.3 mM EDTA at 37 and 49°C resulted in a 2 and 3 log reduction, respectively, in cell survival compared with the 25°C treatment (Fig. 4A). Survival of cells was not significantly affected by treatment in water, nisin, or EDTA alone for 0.3 min at any of the temperatures tested.

Treatment time interacted with temperature. Heat at 49°C for up to 1 min with water alone had no significant effect on survival of bacteria, but with 3- and 10-min exposures, survival was reduced by 2 and 4 log units, respectively. When EDTA + nisin was added to the bath, 1-min treatment reduced survival by 3 log units at 25 and 37°C, and by another log unit at 49°C, from an initial population of over 10^6 CFU/ml (Fig. 4B). At 3 min, 49°C treatments with EDTA + nisin reduced survival to less than 100 CFU/ml.

In vivo inhibition of decay on carrot disks. Based on average disease scores for all categories of carrot types, inoculum levels, and bacteria, soft rot on inoculated carrot disks was reduced by all heat treat-

ments at 45°C and by chlorine (Table 1). EDTA + nisin at either 25 or 45°C did not affect the overall results. Concentrations of EDTA and nisin higher than those used in these tests caused tissue injury and increase in decay susceptibility.

Within the different categories, however, treatment differences were evident. Heat did not reduce lesion diameters on peeled baby carrots, but did on mature carrot disks, and chlorine was effective on both. At low inoculum levels (10^3 CFU per disk), with carrots infected with *P. viridiflava*, decay in all treatments at 45°C was significantly lower than at 25°C. With *P. fluorescens* and *E. carotovora*, only the treatments with EDTA + nisin at 15 and 50 μ g/ml resulted in less decay at 45°C, but only by enough to statistically separate them from 25°C treatments at the 90% level of confidence ($P = 0.10$). Chlorine treatments were statistically better ($P =$

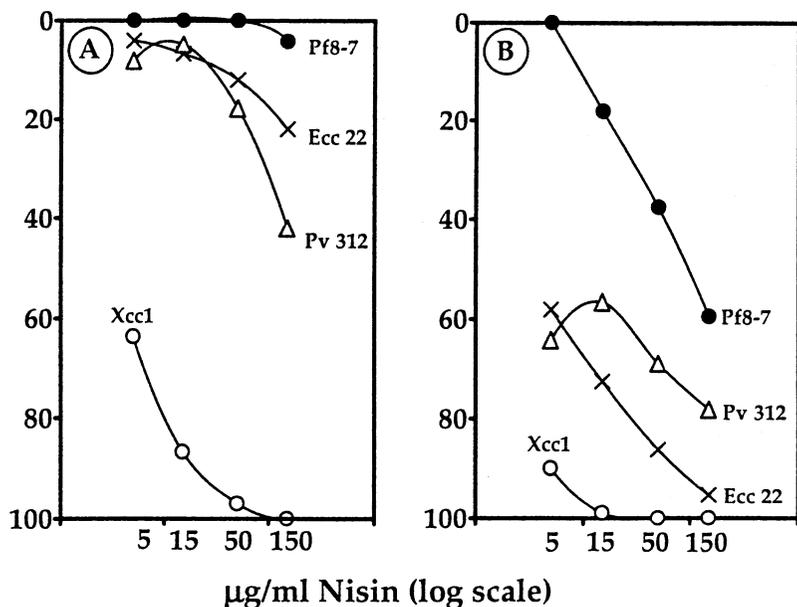


Fig. 3. Effect of EDTA on the inhibition of four soft-rotting bacteria by nisin at 25°C. (A) Nisin at 5, 15, 50, and 150 μ g/ml. (B) Nisin at 5, 15, 50, and 150 μ g/ml plus 0.3 mM EDTA. Each point represents the average of three different levels of inoculum and four time periods, replicated three times. Ecc22 = *Erwinia carotovora* var. *carotovora* 22, Pf8-7 = *Pseudomonas fluorescens* 8-7, Pv312 = *P. viridiflava* SF312, and Xcc1 = *Xanthomonas campestris* 1. (data for *Cytophaga* and *E. chrysanthemi* not shown).

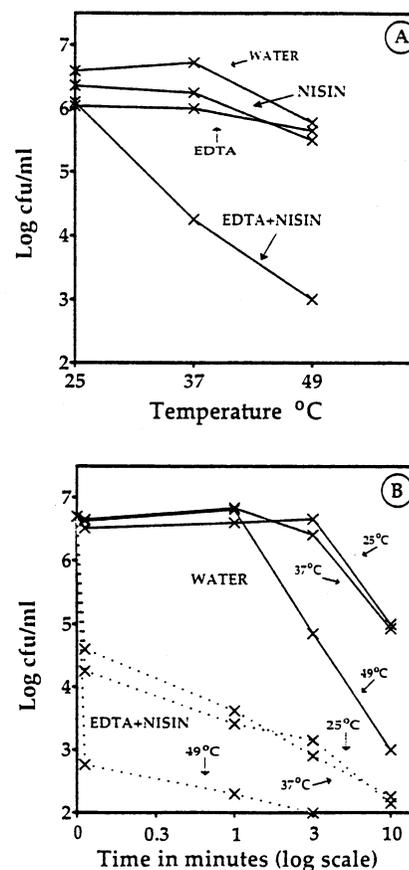


Fig. 4. Effect of treatment temperature and time on survival of four soft-rotting bacteria, *Erwinia carotovora* var. *carotovora*, *E. chrysanthemi*, *Pseudomonas fluorescens*, and *P. viridiflava*, treated with 0.3 mM EDTA and nisin at 15 μ g/ml. (A) Temperature effect on survival with water alone, with 0.3 mM EDTA, with nisin at 15 μ g/ml, and with an EDTA-nisin combination. (B) Effect of treatment time at three different temperatures on survival of the bacteria in water (upper solid lines) and in an EDTA-nisin combination (lower dashed lines). Each point represents an average of three replications at three different inoculum levels for the four bacteria.

0.05) than EDTA–nisin treatments at 45°C only with peeled baby carrots and those infected with *P. fluorescens*. In tests at the high inoculum level (10⁴ CFU per disk), only the chlorine treatments were effective in reducing decay.

DISCUSSION

In vitro responses of the soft-rotting bacteria to EDTA were similar to those previously reported by other authors for pseudomonads and enterobacteriaceae, despite differences in specific assay conditions (6,7,13,19–21). The tolerance of our pectolytic strain of *P. fluorescens* to EDTA was consistent with findings of Payne et al. that concentrations of up to 2.5 mM EDTA had little or no effect on growth (13). *Xanthomonas* and *Cytophaga* were highly sensitive to EDTA, but since their role in postharvest bacterial soft rot is minor and infrequent (11), the information has limited practical significance.

The tissue macerating enzyme PL appeared to be more sensitive to EDTA inhibition in the pseudomonads than in the other bacteria tested. Further research is needed to determine if the cause was inhibition of enzyme production or transport, or an interaction with availability of calcium ions, known to be required for PL activity (8). Yet this fact suggests that EDTA could affect *P. fluorescens* pathogenicity through inhibition of PL if not of growth. Lower disease scores for EDTA-treated carrots infected by *P. fluorescens* (Table 1) tended to support this suggestion. The role of EDTA and heat in enhancing antibiotic activity also suggested an effective combination treatment with an agent such as nisin, which has been an additional advantage of residual activity on adsorbed surfaces (2).

Freshly cut carrots were selected as a host system because they are commercially

marketed and are susceptible to *Pseudomonas* soft rot (personal observations). The use of EDTA, heat, and nisin, within the tolerances of host tissues, resulted in a significant reduction of decay attributable to the heat treatment, but not exclusively to EDTA or nisin. Disease scores tended to be lower for some EDTA + nisin treatments at 45°C, but did not meet statistical tests to distinguish them from 25°C treatments. Further replications may be required to demonstrate their statistical significance at the 95% level. Chlorine at 100 µg/ml, the industry standard, was the best treatment apart from the dry control. The dry control, however, is not a valid comparison since residual moisture associated with liquid treatments was apparently a significant factor in decay development.

The failure of in vivo experiments to closely support results of in vitro testing undoubtedly was due to factors associated with complexities of the host–parasite system. Such factors include tissue defense responses to invasion by pathogens; possible injurious effects of EDTA, heat, and nisin on wound-healing or suberization in freshly cut tissues; and mechanical inaccessibility of some infection sites to therapeutic agents. In addition, cells surviving heat, EDTA, and nisin treatments may have been a significant residual inoculum on the wounded tissues. We did not evaluate growth characteristics of surviving cells, but it may be assumed that they were viable and capable of infection. With further testing on other postharvest host–parasite interactions, the potential of these therapeutic agents may be realized.

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Table 1. Average disease scores^a for various categories of carrot disks inoculated with soft-rotting bacteria, then treated with EDTA and nisin at two different temperatures

Treatment, temperature ^b	10 ³ CFU/disk ^c			10 ⁴ CFU/disk ^d (33)	Carrot types ^e		Average (66)
	Pv312 (21)	Pf8-7 (6)	Ecc22 (6)		Mature (57)	Baby (9)	
Water, 25°C	2.16 a	1.60 a	2.78 a	2.28 a	2.18 a	2.88 a	2.26 a
EDTA (0.3 mM), 25°C	2.28 a	1.34 a	2.82 a	2.72 a	2.46 a	3.02 a	2.54 a
EDTA + nisin 5 µg/ml, 25°C	2.48 a	1.26 a	2.62 a	2.70 a	2.56 a	2.66 a	2.58 a
EDTA + nisin 15 µg/ml, 25°C	2.48 a	1.26 a	2.62 a	2.72 a	2.58 a	2.84 a	2.60 a
EDTA + nisin 50 µg/ml, 25°C	2.42 a	1.46 a	2.66 a	2.70 a	2.54 a	2.80 a	2.62 a
Water, 45°C	1.52 b	1.66 a	2.72 a	1.80 a	1.76 b	2.44 a	1.80 b
EDTA (0.3 mM), 45°C	1.60 b	1.30 a	2.18 a	2.02 a	1.94 b	2.24 a	1.98 b
EDTA + nisin 5 µg/ml, 45°C	1.48 b	1.26 a	2.34 a	1.98 a	1.84 b	2.38 a	1.92 b
EDTA + nisin 15 µg/ml, 45°C	1.30 b	0.96 ab	1.74 ab	2.26 a	1.96 b	2.10 a	1.98 b
EDTA + nisin 50 µg/ml, 45°C	1.28 b	0.60 ab	1.68 ab	2.12 a	1.88 b	2.12 a	1.88 b
Chlorine (100 µg/ml)	1.28 b	0.30 b	1.32 ab	1.58 b	1.46 b	1.04 b	1.42 b
Dry control	1.16 b	0.30 b	0.54 b	1.94 a	1.66 b	0.84 b	1.56 b

^a Based on diameter of soft-rotted area on inoculated disk: 1 = less than 2 mm, 2 = 2 to 10 mm, 3 = 10 to 20 mm, and 4 = over 20 mm. Averages in each column not followed by the same letter are significantly different at 95% level of confidence ($P = 0.05$).

^b Five disks per replication, inoculated with 10-µl bacterial suspension at 10⁶ or 10⁵ CFU/ml (10⁴ or 10³ CFU/disk, respectively), incubated 2 h at 25°C, dipped 1.5 min in treatment solution, then incubated 1 to 3 days at 25°C.

^c Pv312 = *Pseudomonas viridiflava*, Pf8-7 = *P. fluorescens*, Ecc22 = *Erwinia carotovora* var. *carotovora*. Averages based on combined data for mature and peeled baby carrots.

^d Averages based on combined data for Pv312, Pf8-7, and Ecc22, and for mature and peeled baby carrots.

^e Averages based on combined data for Pv312, Pf8-7, and Ecc22.

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