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Effect of sulfur dioxide fumigation on survival of foodborne pathogens on table grapes under standard storage temperature

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ABSTRACT

We examined the fate of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella enterica* Thompson inoculated on freshly-harvested table grapes under standard cold storage with initial and weekly sulfur dioxide (SO_2) fumigation. *L. monocytogenes* and *S. enterica* Thompson were much more sensitive to cold temperature than *E. coli* O157:H7. Furthermore, *L. monocytogenes* was highly susceptible to SO_2. Initial fumigation with 100 or 200 ppm-hr was sufficient to eliminate this pathogen on grapes with low (10^4 cells/grape) and high (10^6 cells/grape) inocula, respectively. Initial fumigation with 300 ppm-hr reduced *S. enterica* Thompson population about 300- and 10-fold on grapes with low and high inocula, respectively. Initial fumigation with 300 ppm-hr reduced *E. coli* O157:H7 population to less than 10-fold, regardless of inoculum density. When grapes were inoculated with the high inoculum and fumigated on days 0 and 7 with 200 or 300 ppm-hr SO_2, *S. enterica* Thompson and *E. coli* O157:H7 were completely inactivated between days 8 and 14 of cold storage. Standard cold storage combined with SO_2 fumigation was effective in reducing and eliminating all three pathogens on table grapes, however, depending on the dose, two or three fumigations were needed for elimination of *S. enterica* Thompson and *E. coli* O157:H7.

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1. Introduction

Fresh fruits and vegetables are increasingly being recognized as important vehicles for transmission of foodborne diseases worldwide. A recent survey of foodborne disease outbreaks in the US during 1998–2008 revealed that vegetables and fruit are among the top four food commodities that cause outbreak-related illnesses (Gould et al., 2013). Among common foodborne bacterial pathogens, *Salmonella*, Shiga toxin-producing *Escherichia coli* (STEC), and *Listeria* are the top etiologic agents associated with hospitalizations and mortality (Gould et al., 2013). Several studies suggest that enteric pathogens have sufficient fitness to survive on plants (Brandl, 2006; Teplitski et al., 2009; Tyler and Triplett, 2008). Their fitness on fresh produce may vary, depending on the biotic and abiotic factors that the pathogens encounter when they colonize the plant surface. Numerous studies on the behavior of enteric pathogens on fruit have focused on cantaloupe and tomato. Although grapes have never been implicated in an outbreak of enteric illness, little information is available about the fitness of enteric pathogens on this fruit during postharvest processes and storage.

Grapes are a non-climacteric fruit that is rich in phytonutrients and antioxidants (Morre and Morre, 2006; Pezzuto, 2008). In California, table grapes are available nearly year-round. Most California table grapes are packaged in the field before storage. Standard industry practice for harvested California table grapes is to perform initial sulfur dioxide (SO_2) fumigation during pre-cooling of the grapes, with doses between 100 and 300 ppm-hr, followed by weekly fumigations with similar doses during cold storage. Ideally, storage rooms should operate at −1.0 to 0.0 °C (30 to 32 °F) with 90 to 95% relative humidity (RH) and moderate airflow, which is important to limit water loss from fruit stems. Fruit should be stored at pulp temperature of −0.5 to 0.0 °C (31 to 32 °F) throughout the postharvest life (Crisosto and Smilanick, 2014). Stem browning and gray mold infection caused by the fungus *Botrytis cinerea* are the two main factors that reduce table grape

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postharvest quality. SO_2 fumigation is an effective way to control the growth of mold on table grapes, thus extending their shelf life (Palou et al., 2002; Smilanick and Henson, 1992), but its effect on enteric bacterial pathogens has not been investigated.

In this study, we investigated the fate of three common enteric pathogens, *E. coli* O157:H7, *Salmonella enterica* Thompson, and *Listeria monocytogenes*, inoculated onto freshly-harvested California table grapes under standard postharvest storage conditions of temperature and humidity. Furthermore, we determined the effect of routine fumigation with standard doses of SO_2 on the growth and survival of these enteric pathogens known to cause serious infections associated with consumption of fresh produce in the US.

2. Materials and methods

2.1. Bacterial strains

A representative strain of *E. coli* O157:H7, *S. enterica* serovar Thompson, and *Listeria monocytogenes* was selected based on isolation source and virulence properties. *E. coli* O157:H7 strain H1827 is a clinical isolate linked to a lettuce-associated outbreak in 1996 in the US (Hilborn et al., 1999). *S. enterica* serovar Thompson strain 99A2342 is a clinical isolate associated with an outbreak linked to cilantro in California (Campbell et al., 2001). *L. monocytogenes* strain 91E01275-9 serotype 1/2b was isolated from a garlic surveillance sample in California (R. Mandrell, unpublished data). A spontaneous rifampicin resistant mutant of *L. monocytogenes* was isolated by plating the overnight culture on LB agar plates containing 100 μg/ml of rifampicin. The rifampicin resistant mutants of *E. coli* O157:H7 and *S. enterica* serovar Thompson were described previously (Brandl, 2008; Brandl and Amundson, 2008; Brandl and Mandrell, 2002).

2.2. In vitro survival test

Single colonies of each pathogen were inoculated in LB broth supplemented with rifampicin at a final concentration of 25 μg/ml and grown at 28 °C overnight on a shaker (150 rpm). At least three biological replicates were prepared for each treatment. Cells were collected by centrifugation at 8000 g for 3 min and washed twice in KP buffer (10 mM K_2PO_4, pH 7.0). Ten micro liter of cell suspension at a concentration of 10^5 and 10^7 cells/ml was spotted onto a Durapore filter (0.2 um pore size) placed on a water agar plate (1.5% agar in deionized H_2O), resulting in a final concentration of 10^3 cells/filter (low inoculum) and 10^5 cells/filter (high inoculum), respectively. The water agar plates were then incubated at 0.2 °C with 95% RH for 14 days in a refrigerated incubator (Fisher Scientific™ Isotemp™ Undercounter BOD Refrigerated Incubator). A relative humidity of 95% was achieved using saturated potassium sulfate solution placed in the incubator. Filters were retrieved weekly to determine the number of surviving cells. Briefly, each filter was placed in 3 ml KP buffer, then cells on the filter were released to the KP solution by sonication and mixing with a vortex using similar parameters applied to release *E. coli* O157:H7 cells from *in vitro* biofilms (Carter et al., 2012). The viable cells were recovered by plating cell suspensions onto rifampicin-containing LB agar plates (50 μg/ml) using an automated spiral plater (Autoplate4000; Spiral Biotech, Norwood, MA). Bacterial concentrations were measured by counting colony forming units (CFU) after overnight incubation at 37 °C. Survival of each pathogen was estimated by comparison of the bacterial counts after incubation under various conditions with bacterial concentrations measured from filters immediately after spotting on Day 0.

2.3. In vivo survival test

Grapes used in the experiments were freshly-harvested California table grapes that were fumigated during the pre-cooling of grapes conducted in a cold storage facility after harvest. Grapes were shipped cold directly from the harvest sites and stored at 4 °C for about 2 days prior to inoculation. On the day of the experiments, grapes were moved to a biosafety cabinet and air-dried for 30 min. Only firm and intact grapes were used for inoculation. Grape berries were placed with pedicel side down on each well of a 6-well or 24-well culture plate (Corning Nos. 3527 and 3473), depending on the size of grapes of a particular batch. Five micro liter cell suspension at a concentration of 10^6 cells/ml and 10^8 cells/ml was spot-inoculated on the surface of grapes, resulting in a final inoculum of about 10^4 cells per grape (low inoculum) and 10^6 cells per grape (high inoculum), respectively. The absolute inoculum concentration was determined by counting colony forming units (CFU) of 10-fold serial dilutions of the cell suspension. At least six biological replicates were prepared for each inoculation concentration. The grapes were then incubated at 0.2 °C and 95% RH for 14 days. The number of surviving cells on each grape berry was determined at various incubation times by plate counts as described above.

2.4. SO_2 fumigation

Bacterial cultures were prepared and both filters and grapes were inoculated as described above. For each inoculation concentration, four SO_2 treatments with a dose of 0, 100, 200, or 300 ppm-hr were applied. The dose was monitored with a Gastec sulfur dioxide dosimeter (Nextteq LLC). Each treatment was carried out in triplicate and six replicates (biological replicates) for cells inoculated on the filters and grapes, respectively. Fumigation was carried out at the onset of the experiment (initial fumigation on Day 0), followed by weekly fumigation and cold storage for two more weeks (Days 7 and 14). For *in vitro* experiments, the effect of SO_2 fumigation on survival of the individual bacterial species was determined by comparing population sizes on the filters following each SO_2 treatment with those on the corresponding control filters prior to fumigation. The effect of SO_2 fumigation on survival *in vivo* was assessed by comparison of population sizes on treated and untreated grapes. An enrichment procedure was also used to detect populations below the limit of detection achievable by direct plating. Briefly, 1.5 ml of the wash solution used to remove cells adhered to the grapes was mixed with an equal volume of 2× tryptic soy broth (TSB) and incubated at 37 °C over-night. The culture was then plated on rifampicin-containing LB agar to detect the presence of the pathogen of interest.

2.5. Statistical analysis

Statistical analysis was performed with SigmaPlot version 11.0 (Systat Software, Inc.). An unpaired t-test was performed for a two-group comparison, and the Analysis of Variance (ANOVA) followed by Bonferroni t-test was performed for multiple comparisons.

3. Results

3.1. In vitro survival of pathogens under cold storage conditions

The response of the three pathogens to the standard cold storage temperature for table grapes was examined under *in vitro* conditions by spotting each pathogen onto a filter with an amount of either 10^3 (low inoculum) or 10^5 (high inoculum) cells per filter