FATE OF *SALMONELLA* INTRODUCED TO CANTALOUPE THROUGH NATURAL FRUIT CRACKS AND FLOWERS, ALONE OR IN THE PRESENCE OF THE PLANT PATHOGEN *ERWINIA TRACHEIPHILA*

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Abstract: *Salmonella*-tainted cantaloupe has been implicated in foodborne illnesses. We know little about *Salmonella* ecology on cantaloupe flowers and fruits, and chose to investigate whether it can enter edible tissues, and whether its interactions with other microbes influence its fate. We assessed the survival and potential internalization of *S. enterica* and the wilt bacterium, *Erwinia tracheiphila,* on cantaloupe after fruit surface or flower inoculation. *S. enterica*, *E. tracheiphila*, or a mixture of the two (10^7 cfu/ml) were introduced onto natural rind cracks or into the flower whorl. Inoculated rind and sub-rind mesocarp were sampled at 0, 9 and 24 days post-inoculation (DPI). Flower samples were collected at 0 and 43 DPI, and interior mesocarp at 15 and 43 DPI. *S. enterica* survived on 40% and 14% of cantaloupe rinds inoculated with both pathogens, or *S. enterica* only, respectively. 58% of *E. tracheiphila* inoculated samples developed watersoaked lesions on rinds. Unlike *S. enterica, E. tracheiphila* traversed some fruit cracks and 31% of sub-rind mesocarps were positive at 24 DPI. At 0 and 43 DPI all blossom samples receiving *S. enterica* alone, or the mixture, were positive for *S. enterica*. At 43 DPI, the populations of *S. enterica* were significantly ($P<0.05$) higher than these at 0 DPI from 4.46 to 6.12 log cfu/ ml and 4.89 to 6.86 log cfu/ml, respectively. *E. tracheiphila* was never detected after day 0. A mesocarp sample from one fruit, flower-inoculated with *S. enterica* only, was positive for this bacterium. The results suggest that *S. enterica* can survive on the rind until fruit maturity. *E. tracheiphila* can traverse the cracked rind, causing watersoaking of interior tissues; the leakage of cell contents can enhance *S. enterica* survival on the fruit surface. Fruit contamination after flower inoculation with *S. enterica* was a rare event under our conditions, but flowers can harbor the bacteria until fruit maturity, thereby becoming a potential reservoir. Use of agricultural practices minimizing fruit contact with potentially contaminated substrates could reduce the risk of *Salmonella* contamination.

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CHAPTER I

INTRODUCTION

Contamination of fresh vegetables and fruits by human pathogens such as *Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Campylobacter jejuni* occurs repeatedly partly because of the successful adaptation of these pathogens to environments associated with food production, processing, and storage. Fresh produce includes a variety of unprocessed fruits and an increasing number of vegetables that previously were consumed predominantly after processing. As consumption of fresh produce has increased, so have incidents of foodborne illness (5, 31, 32). The number of such outbreaks doubled between 1973 to1987 (4) and continues to occur due to fresh produce consumption in the United States. Many health-conscious consumers, wishing to maximize the nutritional content of their food, recognize that less processing often means that more nutrients remain.

Cantaloupes, and then tomatoes, are the most popular raw produce types worldwide, and cantaloupe was the second most implicated type in *Salmonella* outbreaks (1, 6, 12). Among 54% of human illness outbreaks associated with the consumption of fresh produce in which the pathogen was identified, 60% were caused by bacteria, and of these *Salmonella* caused 48% (32). *Salmonella* serovars implicated include Chester in

1990 (28), Saphra in 1997 (32), Oranienburg in 1998 (18), and Poona in 2000, 2001 and 2002 (1). Although plants have not generally been considered a niche for human pathogens, this paradigm is now being reconsidered. Uptake of *Salmonella* after artificial inoculation has been reported to occur in several plant species (2, 16, 20). *Salmonella* was taken up by tomato hypocotyls cotyledons, and stems after inoculation onto previously wounded roots of seedlings grown in a hydroponic system (16). Sources of *Salmonella* in field contaminations can include irrigation water (13, 24, 25), insect vectors [particularly houseflies (17) and other flies (33)], soil and crop debris (3). *Salmonella* survives for as long as 405 days in sterilized manure-amended soils (35).

Very little information is available on the mechanisms of human pathogen internalization in fresh produce (20). Pathogens might enter the plant/fruit through natural openings, such as stomata, lenticels and nectarthodes. Cantaloupe fruit is smooth and hairy until about 10 days after pollination, when the rind begins to crack because of fruit expansion. This process continues for 10-15 days, but the cracks are soon healed by corky growth, which becomes the netting for netted melon types. The cracks are openings through which microflora from sources such as manure, irrigation water or soil might enter. However, pathogen internalization through the cracks has not been documented.

The presence of other plant resident microorganisms, including plant pathogens, can be beneficial for the growth and colonization of *Salmonella* (3, 34). The relationship between any two microbes on the plant surface varies with the plant species, the microbial species, and the conditions, and it could be negative, positive or neutral to the participants. The more positive the relationship becomes, the more difficult it is to remove the microorganisms from the plant surface (19). Barak and Liang (3) showed that

at the 3-5 leaf and pre-bloom stages of tomato plants, *S. enterica* populations were significantly higher after co-inoculation with *Xanthomonas campestris* pv. *vesicatoria* than when the human pathogens were inoculated alone. A synergistic relationship of *Salmonella* spp. with storage fungi was observed during the storage of market vegetables (34), when co-inoculation of tomato fruits, potato tubers and onion bulbs with *Salmonella* Typhimurium and either *Botrytis* or *Rhizopus* resulted in increased populations of *Salmonella* compared to those on control fruits inoculated with *Salmonella* alone. A similar study by Brandl et al. (7) showed possible synergism between *S. enterica* and *Aspergillus niger*, attributed to cellulose-chitin interactions. Similarly, co-inoculation with *Cladosporium cladosporioides* greatly enhanced the ability of *S. enterica* to penetrate (3-4 cm inside the rind) mesocarp tissues of cantaloupe fruit (29).

E. tracheiphila, an important pathogen of most cucurbits including cantaloupe, causes bacterial wilt disease (10, 11, 23). It is naturally transmitted by two cucumber beetles (striped: *Acalymma vittatum*. F. and spotted: *Diabrotica undecimpunctata hawoardi* Barber) (11, 23, 27). It overwinters in adult beetles (11, 14, 26) and transmission occurs when these insects feed on plants and their frass contaminates fresh feeding wounds (8, 9, 21-23, 27) on leaves, stems, or flower nectaries (30). To date, no other means of transmission has been reported. *E. tracheiphila* eventually enters the xylem vessels multiplies, and produces exopolysaccharides, thereby blocking water flow and causing wilting (30).

Our brief report from a preliminary experiment suggests that, following flower inoculation, *E. tracheiphila* is able to colonize cantaloupe fruit and traverse to vines, causing wilting (15). We wanted to investigate whether *S. enterica* could survive and

colonize fruit rinds and flowers, and whether they could gain access to the edible fruit mesocarp from those locations. We also wanted to understand whether *E. tracheiphila* influences *S. enterica* survival or internalization on cantaloupe fruits or flowers. The output of this work will aid our understanding of the relationship between human and plant pathogens on flower and fruit surfaces and will help to identify strategies to reduce fresh produce contamination by human pathogens.

The objectives of this research are to understand survival and internalization of *Salmonella* on cantaloupe, with or without the influence of a plant pathogen:

1- To characterize the survival and internalization of *S. enterica* on or in cantaloupe fruit when inoculated on the rind at the time of natural fruit cracking, alone or in the presence of the plant pathogen, *E. tracheiphila*.

2- To investigate the survival and internalization, of *S. enterica* when introduced into flower interiors, alone or in the presence of the plant pathogen *E. tracheiphila*.

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CHAPTER II

REVIEW OF LITERATURE

I – History, origin and nutritive value of cantaloupe

Cantaloupe (*Cucumis melo*), also known as cantaloup, muskmelon, or rockmelon, is in the family Cucurbitaceae, which includes nearly all melons and squashes. There are two major varieties of cantaloupes. *Cucumis melo* var. *cantalupensis* (grown mainly in Asia and Europe) is considered to be a true cantaloupe as it is rough and warty. *Cucumis melo* var. *reticulatus* is grown largely in the United States, where "cantaloupe" has become a generic name for all kinds of netted, musk-scented melons (64). Cantaloupe is a rich source of nutrients including fiber, minerals, and almost all the vitamins for a normal human health. In a market survey Eitenmiller et al. (25) found that levels of niacin, riboflavin, thiamin, ascorbic acid, folacin and chromium (Cr) in cantaloupe fruit were significantly higher during maximum availability periods than at other times.

The name of the fruit comes from the city of Cantalupo, near Tivoli, Italy, where cultivation began in the sixteenth century (57). Christopher Columbus, on his second voyage in 1494, introduced cantaloupes to the North America. Robinson and Decker-Walters (60) suggested that Asia or Africa could be the origin of muskmelon. Evidence

based on genetic studies, attempts at crossing with other *Cucumis* species, and the worldwide distribution of melon varieties suggests that Africa was the origin (39). However, melon domestication started in Egypt over 3,000 years ago (55). Melon dispersion may have occurred from Africa to the Middle East and Asia, where secondary diversification and domestication development could have occurred (39).

II – Cantaloupe production in the United States

In the United States, California continues lead in melon production, accounting for 43% of the harvested area, 49% of production, and 48% of the value (75). The total United States cantaloupe production in 2011 was 8.55×10^5 metric tons, with a total area of 2.87×10^4 ha. California is responsible for 5×10^5 metric tons of cantaloupe according to the 2011 census (75). Cantaloupe is also produced in Oklahoma on 446 acres (76). Althogh the per capita civilian utilization of cantaloupe has been decreasing since 2001 (75), the fruit remains popular nationwide.

III - Growth conditions

III –a -Temperature

Melons are warm-season annuals that are very sensitive to frost at any growth stage. Seedlings planted in the greenhouse should not be transplanted to the field until the soil temperature (3 inches beneath the soil surface) reaches 60° F. Growth is very slow below 60^oF (16^oC) and the optimum temperature for growth ranges from 85° to 95^oF (30° to 35^oC), although cantaloupe can tolerate temperatures in excess of 104^oF (40^oC). Average base, optimum and upper critical growth chamber temperatures of 49.5^oF, 93^oF and 113^oF, respectively, were established for cultivars Gold Rush and Mission (7). In the field, the crop is best grown on raised beds covered with black or silver plastic mulch to

protect the melons from rotting, a common problem when the fruits are in contact with soil (James Motes, Oklahoma State University, Department of Horticulture, retired; personal communication).

III –b -Soil

Soil texture can be used as an indicator for growers to decide whether to plant early or late in the season (33). Sandy soils are suitable for early plantings because of their more rapid heating. Loam and clay loam soils are preferred for mid-season production because of their high water-holding capacity as they prolong the harvest period, thus making fruit available throughout the season. Irrespective of soil texture, cantaloupe can be grown on any soil provided that it is well drained.

III –c -Irrigation

Yield and income can be maximized with wise selection of cultivation techniques and the appropriate amount of water for fruit growth and development (1). In spite of the fact that furrow irrigation could increase the microbial contamination of fresh produce including cantaloupe compared to sub-surface irrigation (66), furrow irrigation is commonly used for its economy and simplicity (48). A total of 2-5 irrigations/season are generally adequate (but frequency of irrigation also depends on rainfall amounts) after the establishment of the crop and the last irrigation should be given 7-10 days before harvest. Drip irrigation is gaining popularity as it is easy to do, uses water efficiently, and results in less foliar and fruit disease than with overhead irrigation. Furthermore, drip irrigation does not interfere with the activity of honeybees in pollination and fertilization. A combination of drip irrigation and plastic mulch is the best for highest fruit yield (18, 42), and reduces water requirements as well as insects, pathogens and weeds (18).

Mohamedien et al. (11) found perforated tunnels with polyethylene mulch treatment resulted in taller plants, higher and earlier yields and thicker fruit flesh.

III-d –Fertilizer requirements

Cantaloupes are heavy users of soil nutrients. Average fertilizer application rates are 90 to 168 kg ha^{-1} (80-150 pounds per acre) of nitrogen (N) and 45 to 225 kg/ha (40 to 200 poundsper acre) of P_2O_5 and potassium, depending on the nutrients available in the soil (33). Higher doses of phosphorus promote fruiting and optimum amounts assure sweetness. The peak period of nutrient absorption in cantaloupe production, 44 days after transplanting, coincides with the period of highest fertilizer demand (4). Macronutrient requirements of cantaloupe fruits are, in order, K> N> Ca> P> Mg> S, corresponding to 46.7, 29.5, 11.3, 4.7, 4.5, and 4.0 g kg^{-1} dry matter, respectively (67). In one study, N accumulated in the vegetative parts such as leaf and stem whereas P and K accumulated more in the fruit (67). Macronutrients, if given in adequate amounts, lead to optimum plant development and fruit yield.

IV-Pollination and fruit development

IV-a -Pollination and fruit set

Cantaloupe plants produce male, hermaphrodite, and female flowers (the latter, rarely) and they need insect activity for pollination and fruit set. Cantaloupes are pollinated mostly by honeybees during the early hours of the day when the flowers are open. Farmers maintain hives to assure high yields and large melon size. Pollination can occur over a period of a week after flowering without adverse effect on harvest productivity (24). Growth of pollen tubes within the stigma is favored by pollination of newly opened flowers (77). Post-pollination, fruit setting can be inhibited by the presence

of other fruits on the same vine. Better fruit set results from insect pollination (70%) than from hand pollination (40%) (45). Only 1-4 fruits per vine will mature (27, 37). Pollen non-viability and self- or cross-sterility can be problematic in some cantaloupe varieties (9). Artificial (i.e. hand) pollination, done by collecting pollen from male flowers and rubbing it onto the stigma surfaces, is practiced for greenhouse grown cantaloupes.

IV-b -Rind development

The surface of the newly formed fruit is always smooth and hairy with a waxy cuticle. Netting generally starts towards end of the fruit-expansion stage (38) but natural surface cracking begins when the fruit is around 10-12 days old (Benny Bruton, USDA, Lane, OK, retired; personal communication) usually near the blossom scar (38). Cracking results from short periods of epidermal cell division. The cracks increase in number and length as the fruits mature, and the fruit surfaces are covered with cracks by 21 days post-anthesis (79). The familiar netting of cantaloupes is due to the deposition of a corky layer derived from a sub-epidermal periderm, which has been characterized as an elaborate system of lenticels. Netting gives roughness to the fruit surface, providing numerous pockets that can serve as shelter to various microflora and create vulnerability to microbial contamination. Netted rinds are difficult to sanitize (71, 74). As low as 150 bacteria cm^{-2} present on netted cantaloupe rind surface can contaminate the edible mesocarp upon cutting (3, 47, 71, 77).

V –Varieties and performance

Some cantaloupe varieties grown commonly in the United States include Ambrosia, Burpee Hybrid, Classic, Cordele, Gold Star, Imperial 4-50, Mainstream, Magnum 45, Mission, Saticoy, Summet, and TAM-Uvalde. Varieties that perform well in

Oklahoma include Caravelle, Cruiser, Sugarcube, Rockstar, Athena, Ambrosia, Super 45 and PMR 45 (James Motes, Oklahoma State University, Department of Horticulture, retired; personal communication). Most of the latter varieties are netted and weigh between 2 lb to 6 lb. Sugar Cube, a new, compact, "personal-size" (4" diam) hybrid from Seneca Vegetable Research (Flat Street, NY) has deep orange flesh, good taste, and excellent storage life. This variety also is resistant to many diseases of melon (29). Edible flesh ranges from pink to orange in color and the rind has pronounced netting. The shelf-life of cantaloupe (either American or British type) compared to other melon types is intermediate to poor (6- 12 days) among six different varieties (*acidulous*, *cantalupensis*, *inodorus*, *saccharinus*, *reticulatus* and an unknown variety) tested (43). Moreover their plant height, fruit weight and total soluble solids are also affected by growing conditions.

VI-a-Problems related to fresh produce consumption

VI-a-i-Outbreaks of salmonellosis

Outbreaks of human illness related to consumption of uncooked fresh produce, contaminated with human pathogens in the form of raw fruits and vegetables or juice, have led to food poisoning and death. From 1973 to 1997 in the United States, 190 fresh produce-associated disease outbreaks were reported with 16,058 illnesses, 598 hospitalizations and eight deaths (65). Human pathogens associated with fresh produce include bacteria, protozoa, and viruses. *Salmonella* was associated with 48% of the bacterial disease outbreaks. Among three multistate outbreaks of *Salmonella* infections, two were associated with consumption of cantaloupe and one with watermelon. Among recent outbreaks related to fresh produce, cantaloupe was the second most implicated produce type, after tomato (10, 26). Cantaloupe has been a common vehicle of

Salmonella contamination. *S. enterica* serovar Chester was named in a 1990 outbreak in which 245 disease cases were registered in 30 United States (68). Because reported cases usually only a fraction of the total number, actual numbers are likely much higher (49).

VI-a-ii-Pre-harvest contamination

Cantaloupe fruit contamination can take place at any point from field production to consumption. Irrigation water and animal manure have been common sources of field contamination by human pathogens. Among various types of irrigation, sub surface drip irrigation may be safest for less contamination (70). *Salmonella* and hepatitis A virus can survive even 14 days after the last irrigation in the field (70). In one field survey conducted to assess microbial quality of fresh produce, *Salmonella enterica* serovar Montevideo was detected in 0.8% of all produce studied, and in 3.3% of cantaloupes (36). *Salmonella* colonized plant roots at higher populations than did *Escherichia coli* (23). Dominance of this pathogen on alfalfa sprouts has also been reported (8), and *S. enterica* was more capable of attachment to alfalfa sprouts than *E. coli,* even after several washings. *Salmonella* survives in a variety of different agricultural environments depending upon the availability of nutrients and a conducive soil pH (35). Moreover, *Salmonella* can survive as a resident on the surface of fresh produce at the time of fruit harvest. Recoveries of *Salmonella* from stomached produce were highest, although not significantly so, and those from homogenized produce were lowest.

Salmonella survival on plant surfaces, and in soil, manure and irrigated water has been well studied. Abiotic factors such as temperature, moisture and soil type may impact bacterial longevity. *Salmonella* survived for 45 days in wet soil (30), 231 days in poultry

compost-amended soil (35), 150 days in almond orchard soil (22), 77 days in loamy sand (19), 3 years in animal feces (53), and 405 days in manure-amended sterilized soil (81).

VI-a-iii-Post-harvest contamination

Fruits and their products post-harvest, can act as vehicles for human pathogens, if not properly handled (46, 56). Fresh produce sampled in the packing shed can have significantly higher levels of microbial contamination than that sampled on the farm (2), suggesting improper postharvest handling. Seasonal differences can also affect produce contamination, which was higher during the fall months, i.e. September, October, and November, than at other times of the year. The type of fresh produce also influences the risk of contamination; after artificial inoculation with human pathogens, cantaloupe supported bacterial growth and multiplication for longer periods of time than did lettuce and bell pepper (69).

VI-b-Association of human pathogens with fresh produce

VI-b-i-Affinity of *Salmonella* to fresh produce

Specific serovars of *Salmonella enterica* associate preferentially with specific fruits or vegetables. For example, *Salmonella* Montevideo was the most persistent on tomato, with higher recovery numbers on tomato fruit surfaces than were recovered with serovars Poona and Michigan. Serovars Hartford and Enteritidis had little to no attachment under these conditions (30). Serovar Chester was linked with an outbreak related to contaminated cantaloupe in the United States in 1990 (68). Another three outbreaks attributed to *S*. Poona have been epidemiologically linked to cantaloupes grown and imported from Mexican farms (5, 26), where iguanas had been feeding on melons in the field. Pet iguanas can be reservoirs of *S.* Poona infection in children (5).

Salmonella Saphra caused another outbreak of foodborne illness that was blamed on imported cantaloupe from Mexico (51). Other serovars of *Salmonella* also establish specific interactions with particular species of fresh produce (5, 26, 52).

VI-b-ii-*Salmonella* internalization

Associations of human pathogens with fresh produce have generally been reported as surface contamination, but recent reports have suggested the possibility of internal contamination (31, 32, 40). Internalization, or movement of the pathogen from the surface to the interior of the plant, has significant implications for the effectiveness of sanitizing procedures and the level of health risk. On artificially inoculated iceberg lettuce leaves exposed to light, *Salmonella* cells clustered near open stomata, entered into leaves via the stomatal openings, and remained close to photosynthetically active cells (40). However, internalization did not occur when plants were kept in darkness and discontinued photosynthesis, suggesting that *Salmonella* may be attracted to plant cells that are activated by light (40). A positive effect of light on *Salmonella* motility was noted.

Salmonella was borne internally in tomato, both after infiltration through the stem scar at harvest (31) and in artificially inoculated tomato plants grown in a hydroponic system (32). Among several tested serovars, Montevideo and Poona had the highest rates of internalization. However, the percentage of fruit having contaminated pulp (55%) was lower than that of fruit surface (82%) or stem scar (73%) contamination (31).

Though many reports of cantaloupe root and fruit surface contamination have been published, there has been no report of *Salmonella* internalization in cantaloupe (23, 44). Therefore, one objective of this study was to address whether *Salmonella* Poona can

enter and colonize cantaloupe fruit interiors through natural flower openings, such as nectarthodes, stigmas and pollen tubes, or through natural cracks on fruit surfaces.

VI-c-*Salmonella* behavior on cantaloupe rind

Biofilms are matrix-enclosed bacterial populations in which bacteria are in contact with each other and with the substrate. Biofilms are formed by both plant pathogens and human pathogens on plant surfaces, including the rind of cantaloupes (6). *Salmonella* Poona RM 2350 and *S.* Michigan formed biofilms within two hours after inoculation on to cantaloupe rinds at 20°C (6). Embedded in the biofilm's extracellular polymeric material was a fibrillar substance that may serve as a protective shield for these human pathogens against the action of commercially available sanitizers. *Salmonella* serovars Enteritidis, Virchow, Thompson, Typhimurium and Newport produced strong biofilms on cantaloupe rinds while Hadar, Poona and Amager produced weak biofilms (41).

Some bacterial genes associated with biofilm formation by *Salmonella* have been identified. *S. enterica* Typhimurium genes *mIrA* and *adrA* are required for both cellulose production and biofilm formation in LB (complex) medium, whereas STM1987 (GGDEF domain, containing protein A, *GcpA*) is required for biofilm formation in medium devoid of nutrients (28).

VI-d-Interaction of *Salmonella* with other microflora on fruit rind

VI-d-i-Soft rotting bacteria

The survival of human pathogens on fresh produce can be enhanced in the presence of other plant resident human pathogens or plant pathogens. Among forty eight different types of healthy and soft rotted vegetables and fruits tested for presence of

Salmonella, 33% and 30% of the enriched broth and wash samples, respectively, yielded black colonies characteristics of *Salmonella* on XLD plates (80). Soft rot affected specimens had a higher prevalence of *Salmonella* (59% in 533 samples from broth enrichment and 66% in 401 samples from wash water) compared to healthy samples (30% of 402 samples from broth enrichment and 33% in 781 samples from wash water).

VI-d-ii-Storage and pathogenic fungi

No significant difference was found between the populations of *Salmonella* Typhimurium on healthy and injured cantaloupe fruit surfaces, but the *Salmonella* recovery was higher in the presence of rotting fungi than on healthy fruit (26.4% vs. 20.2%) (80). *Salmonella* multiplied to greater titers on fruit surfaces in the presence of *Botrytis* or *Rhizopus,* but to lower titers in the presence of *Alternaria* or *Geotrichum*, as compared with the control. When *Salmonella* Typhimurium was co-inoculated with *Rhizopus* sp. onto cantaloupe surfaces during cold storage, high CO_2 concentration and adverse temperatures decreased *Salmonella* populations slightly, but the presence of *Rhizopus* did not affect *Salmonella* survival (61). However, co-inoculation with *Cladosporium cladosporioides* greatly enhanced the ability of *S. enterica* to penetrate mesocarp tissues of cantaloupe fruit compared to *S. enterica* inoculated alone (59). *S. enterica* Poona grew 3-4 cm below the inoculated cantaloupe rind, following wounding, and moved into the mesocarp when *C. cladosporioides* was present in the co-inoculation treatment.

VI-e-Use of sanitizers in produce processing

Cantaloupes are generally washed to remove surface contaminants before being packaged. In the United States, packaging procedures vary from state to state. For

example, Georgia grown cantaloupes are first moved to packing sheds, where they are washed and then packed, whereas California grown cantaloupes are packed in the field without disinfection. *Salmonella* populations on Georgia grown cantaloupes that were washed in either cold or hot water were reduced by about 0.5-log (3). Even so, *Salmonella* was detected in the rinstate of 1 out of 900 cantaloupe fruits. The use of chlorinated water in packinghouse disinfection tanks did not completely eliminate fungi, total aerobic bacteria and total coliform bacteria from cantaloupe rinds (47). Despite the disinfection practices, *Salmonella* multiplied approximately ten fold, suggesting that it can re-infest the fruit rind after disinfection (47).

Chlorine can be effective in controlling surface-resident foodborne pathogens; 8.0 mg/liter of ClO_2 of gaseous chlorine dioxide reduced *Salmonella* on raspberry by as much as 1.5 log CFU/g (72). Different combinations of sanitizing chemicals reduced *Salmonella* populations on cantaloupe rind surfaces. A 2% commercial detergent formulation (DECCO Apl Kleen 246) followed by 5% H_2O_2 at 50^oC reduced *Salmonella* in excess of 3 logs (62). Application of H_2O_2 as a sanitizer on the rind surface extended the shelf life of cut cantaloupe, killing almost all the bacteria on the melon surface without contaminating the cantaloupe flesh (62).

Uniform glow discharge plasma (OAUGDP) also has been effective in inactivation of human pathogenic bacteria on apples, cantaloupe and lettuce (21). *Salmonella* populations were reduced by >2 log on cantaloupe rind surfaces after one minute exposure to OAUGDP. Chemical sanitizers such as chlorinated or ozonated water or commercial detergents have been used to remove human pathogens from fresh fruits and vegetables, but treated produce may become more vulnerable to human pathogens

after treatment (73). Chlorine (200 ppm), hydrogen peroxide (2.5%) and hot water (96°C) removed *Salmonella* from cantaloupe surfaces, but after re-inoculation with *Salmonella*, pathogen recovery was greater from hot water-treated cantaloupe than from untreated, chlorine or hydrogen peroxide treated fruits (73). The increased probability of re-contamination of sanitized produce compared to that for un-sanitized produce, which may be due to the removal of competing microflora, suggests that sanitizing procedures may have unintended consequences and should be carefully evaluated.

VI-f-Bacterial wilt, insect vectors, and *Enterobacteriaceae*

Muskmelon is susceptible to various economically important viral, bacterial, mycoplasmal, and fungal diseases (82). Bacterial wilt, caused by *Erwinia tracheiphila,* is an important disease, especially in warm climates. Most cantaloupe varieties are susceptible to this disease, which can cause significant losses if the insect vector is present. Muskmelon cvs. Legend and Superstar, among six cultivars tested, had some resistance to *E. tracheiphila,* but placing inocula onto leaves prior to wounding and creating larger wounds on the leaves led to higher infection rates even in the resistant cultivars (13). Pumpkin seedlings were also susceptible to *E. tracheiphila* when artificially inoculated on wounds at the cotyledon stage (12). It has been reported that this pathogen is active only when it invades xylem vessels and is not capable of causing disease epiphytically or through soil medium (58). But according to a recent study, *E. tracheiphila* was able to internalize through male flowers easily in the absence of nectar and caused 48% of plant wilting compared to only 12% plant wilting in the presence of nectar (12%) (63). When ingested by xylem feeding cucumber beetles, the wilt bacteria

are carried to the guts, where they can overwinter and be passed into the frass, later being deposited on floral organs from which they can access the plant system (50).

The striped cucumber beetle (*Acalymma vittatum* F.) is an important vector of *E. tracheiphila* in cucurbit growing areas around the world. Although a single contaminated beetle was not sufficient to transmit the wilt pathogen, significant wilt occurred at beetle densities of 4 or 5 per plant (15). Feeding preferences of the striped cucumber beetle influence the incidence of wilt among cantaloupe varieties grown in the field (16). The beetle's ability to transmit *E. tracheiphila* depends on the total feeding time (14). When the insects fed continuously for 12 h, 24-48 h, or 72 h, only 0.05%, ≈2% and 5% of the beetles, respectively, transmitted *E. tracheiphila*.

Relationships among insects, *Enterobacteriaceae* and cucurbits have been explored. Squash bugs (*Anasa tristis*, De Geer) harbor and transmit *Serratia marcescens*, the causal agent of cucurbit yellow vine disease, on watermelon, cantaloupe, and squash (17). *S. marcescens* overwintered inside the squash bugs and transmitted the CYVD pathogen the following season (17). *S. marcescens* was retained by the insects after 21 days of feeding (54), and continued to transmit after molting (78). Female bugs were more efficient transmitters than males.

Salmonella can be transmitted also by other insects that visit agricultural fields. Flies acquired *Salmonella* when confined in a room containing chickens challenged with *Salmonella enterica* serovar Enteritidis (34). *Salmonella* also can be internally transmitted from one bird to another by the lesser mealworm beetle (*Alphitobius diaperinus,* Panzer) (20). Nearly a decade ago *Salmonella* uptake by tomato fruit after artificial inoculation of its flower was observed (31). There is a need to determine

whether *Salmonella* can be acquired and transmitted by other insects during normal activities such as landing, feeding, and oviposition.

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CHAPTER III

SURFACE SURVIVAL AND INTERNALIZATION OF *SALMONELLA ENTERICA* THROUGH NATURAL CRACKS ON DEVELOPING CANTALOUPE FRUITS, ALONE OR IN THE PRESENCE OF THE MELON WILT PATHOGEN *ERWINIA TRACHEIPHILA*

Abstract

Outbreaks of foodborne illness attributed to the consumption of *Salmonella*-tainted cantaloupe (*Cucumis melo* var. *reticulatus*) have occurred repeatedly. However, we have limited understanding on the ecology of *Salmonella* on cantaloupe fruit surfaces. In this study, we investigated the interactions between *S. enterica* Poona and *Erwinia tracheiphila* on cantaloupe fruit surfaces. Cantaloupe fruits were inoculated, at their natural cracking stage, with these two pathogens, either singly or in a mixture, at 20 µl of 10^7 cfu/ml and spread over 2 x 2 cm of the marked rind surface. Microbial and microscopic analysis of the rind layer was performed at 0, 9 and 24 days post inoculation (DPI). At 24 DPI (fruit maturity), *S. enterica* was still detected on 40% and 14% of fruits inoculated with both pathogens or with *S. enterica* only, respectively (P = 0.11, Fisher's Exact Test, one tailed). Two of the rind samples, inoculated with the mixed culture

treatment, yielded countable *S. enterica* at fruit maturity (24DPI) *E. tracheiphila*, when inoculated alone, internalized through the fruit cracks, causing watersoaking (61%) and traversed to the underlying sub-rind mesocarp (31%) at 24 DPI. *Salmonella* can survive on the cantaloupe surface until fruit maturity when introduced at the time of natural fruit cracking and its survival was enhanced by the presence of *E. tracheiphila.* In this work, *S. enterica* was not detected in the fruit interior, but since *E. tracheiphila* internalized through natural cracks on developing fruits, the possibility that human pathogens might also do so needs further investigation. Good agricultural practices that avoid fruit contact with soil, use of contamination free water and measures that keep plants free of pathogen attack could reduce the risk of *Salmonella* contamination and persistence on the fruit.

Introduction

The occurrence of human pathogens on fresh fruits and vegetables and the incidence of foodborne illness have been increasing in the United States and around the world (34, 63, 64). *Salmonella enterica,* causal agent of salmonellosis, is one of the most common human pathogenic bacteria contaminating fresh produce world-wide (7). Among recent *Salmonella*-associated disease outbreaks, cantaloupe (*Cucumis melo* var. *reticulatus*) was the second most implicated produce type (3, 10, 25). The first documented salmonellosis outbreak, caused by consumption of salad bar cantaloupes contaminated with *S. enterica* Chester in 1990, involved 245 reported cases in 30 U.S. states (58). Since reported cases are only a fraction of the actual number of people sickened, hundreds of illnesses reported could actually indicate thousands or more (46). Cantaloupe fruit is characterized by pronounced rind netting, which contains micro

pockets likely to shelter microflora and prevent effective sanitation (1, 45, 68, 69). As few as 150 bacteria cm^{-2} on the netted rind surface can contaminate the edible mesocarp upon slicing (68).

Cantaloupe fruit netting begins at the blossom scar (40) with natural cracking of the rind on 10-12 day old fruits (47). The cracks lengthen and cover the whole fruit surface at the end of the fruit-expansion stage (40). Stomata present on the fruit surface become nonfunctional with time. Corky surface ridges, consisting of a thick cuticle (40) containing lenticels, which function in gas exchange, form, sealing the cracks (47).

As the rind cracks begin to form, defensive compounds are produced by the plant to reinforce structural and chemical barriers against the threat of pathogen attack (40). Cantaloupe fruits usually develop on the soil surface, where the physical defensive barriers may be compromised, providing a route of entry for saprophytes or plant- or human-pathogenic microbes present in the agricultural environment. Rot is very common in cantaloupes that develop on soil surfaces (James Motes, Oklahoma State University, Department of Horticulture, retired; personal communication).

Human pathogens such as *S. enterica* can be brought into the agricultural field by contaminated irrigation water (27, 49, 53), insect vectors (35), or soil and crop debris (5), and contaminate the growing plants (17, 20, 30, 37, 52, 71). Bacterial uptake and translocation by and within plant parts following artificial inoculation has been reported in many plant species (5, 21, 32, 42). Although the ecology of *S. enterica* on plant surfaces, outside of its mammalian hosts, is poorly understood, several groups have shown that the presence of other plant resident microorganisms, such as soft rot bacteria (72) and storage fungi (60, 62, 71), can promote the growth and colonization of plants by

S. enterica. Fruits having wounds or contaminated with other microflora were more likely than healthy fruits to be colonized by this pathogen. Barak and Liang (5) reported significantly higher *S. enterica* populations after it was co-inoculated with *Xanthomonas compestris* pv. *vesicatoria* onto tomato plants at the 3-5 leaf and pre-bloom stages, than when it was inoculated alone. Similar synergism was reported between species of *Rhizopus* or *Botrytis*, both of which cause rots in vegetables, and *S. enterica* Typhimurium (71). Brandl et al. (11) showed synergism (attachment and biofilm formation) between *S. enterica* and *Aspergillus niger,* possibly due to cellulose-chitin interaction. Pre-incubation of *S. enterica* with N-acetylglucosamine (a monomeric component of chitin) or its cellulose-deficient mutant failed to attach to the fungus. Similarly, co-inoculation of *S. enterica* with *Cladosporium cladosporioides* greatly enhanced its ability to penetrate the mesocarp of cantaloupe fruit (60).

Limited information is available on the possible internalization of either plant or human pathogens through openings created on the cantaloupe rind surface at the time of cracking, and on the possible interactions between plant and human pathogens. Therefore, the objective of this study was to investigate the survival and internalization of the human pathogen, *S. enterica* Poona, and the plant pathogen, *Erwinia tracheiphila* (cause of cucurbit bacterial wilt), on cantaloupe inoculated at the time of natural fruit cracking. We investigated whether the presence of *E. tracheiphila* would influence *Salmonella'*s capacity for long term survival on the fruit surface and on its internalization into the edible fruit mesocarp. The results of this work will help to identify strategies to limit contamination and internalization by human pathogens on this popular and nutritious fruit.

Materials and Methods

Bacterial strains, labeling, storage and inoculum preparation. *Salmonella enterica* Poona from our laboratory collection, a clinical isolate from 2001 cantaloupe outbreak, was plasmid-labeled in our laboratory with pUC18T-mini-Tn7T-Gm-dsRedExpress (fluorescing red) having gentamycin and ampicillin resistance genes following the protocol of Choi and Schweizer (15). *Erwinia tracheiphila* (*Et*) strain MCM1-1, isolated originally from Oklahoma cantaloupe by B. Bruton (USDA-ARS, Lane, OK) and provided by M. Gleason (Iowa State University, IA) was transformed with pGFPuv (Clontech Laboratories, Inc., CA) by electroporation as described in Ma et al. (2) and colonies were selected after growing on ampicillin amended nutrient agar plates. Plasmid stability tests were performed for both labeled pathogens by ten successive transfers in Luria Bertani broth (LB) followed by plating on nutrient agar plates (NAP) or LB agar plates for *E. tracheiphila* and *S. enterica,* respectively. *E. tracheiphila* colonies were observed under UV light and *S. enterica* colonies were observed normal light after 2 days, as they took time to develop fluorescence. Both pathogens were stored in Luria Bertani (LB) broth aliquots, amended with 25% glycerol, at -80°C. For use in experiments, *S. enterica* and *E. tracheiphila* were grown on LB agar amended with gentamycin ($LB_{gent.}$), and nutrient agar amended with ampicillin-$NAP_{amp.}$), at 37°C and 28°C, respectively, for 48 hr. Bacterial cells were harvested with a sterile plastic loop and dispersed well in 0.1% peptone water to a final homogenous suspension of ca. 2 x 10^{7} cfu/ml, determined by optical density (OD) at 600 nm. To prepare mixed strain inoculum, equal volumes of each bacterial suspension were mixed

to yield a final concentration of ca. 10^7 cfu/ml. The inoculum titer was determined by plating appropriate dilutions (in 0.1% peptone water) on agar plates.

Plant management. Seeds of cantaloupe (*Cucumis melo* var. *reticulatus*), cv. Sugarcube, were sown 1" deep in cells of polypropylene flats containing Redi-earth potting mix (SUNGRO®, Bellevue, WA) and placed in a growth chamber (75°F, 60% humidity,14h day/10 h night). Seedlings (21 days old, 2-3 leaf stage) were transplanted to 4.2 gal pots containing Metromix-300 potting mix (Sun Gro, WA) supplemented with slow-release Osmocote fertilizer (19N, 6P and 12 K). Pots were transferred to the greenhouse, where average temperature and humidity were 23°C and 52%, respectively. Greenhouse temperatures were set at 24° C (day) and 18° C (night) with 14 h day/ 10 h night periods.

A week after transplanting, vines were trailed up and tied onto a framework of polyvinyl chloride (PVC) pipes to minimize plant-to-plant contact and to facilitate sampling from identifiable plants. Pots were watered every other day. Pistillate flowers were pollinated, using a fine artist's paint brush, with pollen collected from 1-2 staminate flowers of the same plant. Resulting young fruits were attached to the PVC frame so that, after inoculation, they were free from contact with other plant parts or PVC frame.

Experimental design. Each cantaloupe plant was allowed to produce 2-3 fruits. Fruits of 8 plants were inoculated with each of the three pathogen treatments (*E. tracheiphila* or *S. enterica* or a mixture of the two pathogens) (24 plants), and three plants were inoculated with 0.1 % peptone water as controls (24+3=27 plants per replication) (Figure III-1). Fruits of three plants per treatment (9 plants) were sampled at 0 and 9 DPI, and fruits of five plants per treatment (15 plants) were sampled at 24 DPI. With 27 plants

in each of three replications, a total of 81 plants were sampled in the experiment. Rind, sub-rind mesocarp and inner mesocarp were sampled as illustrated (Figure III-1). Each plant was allowed to produce one additional fruit that received no inoculation, to investigate the systemic movement of the inoculated pathogens; only inner mesocarps were sampled on these plants (Figure III-1). Each treatment consisted of three replicated trials that were conducted from February to September of 2011.

Inoculation of fruit rind. Twelve-day-old fruit, having fresh natural cracks, were inoculated with ca. 10^7 cfu/ml of bacterial suspension. A total of 20 µl of suspensions of *S. enterica*, *E. tracheiphila*, a mixture of both bacteria, or 0.1% peptone (control), were deposited in 10-15 droplets onto the rind within a 2 x 2 cm square drawn with an indelible marker around a freshly formed crack on a single fruit/plant (Figure III-2). The droplets were spread over the marked area using a soft, sterile plastic bristled brush.

Fruit sampling and microbiological analysis. Fruit were sampled immediately after inoculation (0 DPI), at 9 DPI and at fruit maturity (when fruits easily detached from peduncles, averaged as 24 DPI). Fruit sampled at 9 DPI and at maturity were checked, after inoculation and before microbial analysis, for any change in the appearance of the inoculation site. The marked squares were slightly larger at these sampling dates than at the time of inoculation because of the fruit growth. Fruit rinds (2 x 2 cm^2, 2-3 mm thick), associated sub-rind mesocarp (~2 cm thick and 7-10 g weight) from the region immediately underneath the inoculation site, and the inner mesocarp (including ca. 25% seeds by weight) from the center of un-inoculated fruits were analyzed for the presence of both pathogens. Rind layers and sub-rind mesocarp samples were excised aseptically from the pathogen(s) or peptone inoculated 2 x 2 cm squares, whereas inner mesocarp

samples were excised from the whole non-inoculated fruit. A rind fragment 3 cm^2 and 2-3 mm thick was used for microbiological analysis (cultivation and enumeration of viable microbes and PCR) and the remaining 1 cm^2 was processed for analysis under CLSM and SEM (Figure III-1). If the rind sample had any symptoms then that portion was included in all microbial assays and electron microscopic examination. If no symptoms were observed then the 1cm^2 rind piece was excised from a corner of the 2 x 2 cm rind piece.

Rind pieces (3 cm^2) were placed into sterile whirl-pack bags (7 oz., Nasco, WI) containing 10 ml Universal Pre-enrichment Broth (UPB) (Becton, Dickinson and Company, MD) and hand massaged from the outside with firm pressure for 2 min followed by 1 min of vigorous hand shaking. Sub-rind mesocarp samples excised from immediately below the inoculation site, and 25 g of inner mesocarp from the center of un-inoculated fruit, were placed in a whirl-pak bags with filters (24 oz. and 55 oz. capacity, respectively) and macerated with a rubber hammer. UPB was added at a ratio of 1: 9 (wt.: vol.). A 100µl volume of each rind layer and mesocarp homogenate was plated (two replicates) on NAPamp and XLD for enumeration of microbes present at high titers, and 250 µl volumes of the same aliquots were plated on each of 4 XLD and 4 $NAP_{amp.}$ plates for enumeration of microbes present at low titers. XLD plates, specific for *Salmonella* Poona, were incubated at 37°C for 24 h, and NAP_{amp}, selective for GFPuv tagged *E. tracheiphila*, were incubated at 28°C for 3-4 days. The remaining suspensions were incubated at 28°C for 24 h, and then loopfuls of the enriched UPB were streaked onto XLD and NAP_{amp} plates and incubated at 37 or 28° C for 24 h or 3-4 days, respectively. To enrich selectively for *S. enterica*, 100 µl of the overnight enrichment culture was transferred to 10 ml of Rappaport Vasilliadis Broth (RV) (Becton, Dickinson and

Company) and incubated at 42° C for 48 hrs. A loopful of incubated RV broth was streaked onto XLD plates and incubated for 18-24 h at 37°C to observe black colonies that were presumptive of *Salmonella* Poona.

PCR confirmation of *S. enterica* and *E. tracheiphila*. One-ml aliquots of overnight incubated rind and mesocarp samples were centrifuged (5800 x *g* for 10 min) and the pellets stored at -20°C until the DNA was extracted for PCR. DNA was extracted from the frozen pellets using a DNeasy Blood and Tissue Kit (QIAGEN Group, Austin, TX). Pathogen presence was assessed by a multiplex PCR using *Salmonella* specific primers (forward- 5' GTGAAATTATCGCCACGTTCGGGCAA 3' and reverse- 5' TCA TCGCACCGTCAAAGGAACC 3') to amplify a 284-bp nucleotide sequence within the *invA* gene (55) and *E. tracheiphila* specific primers ETC1 (5'GCACCAATTCCGCAGT CAAG3') and ETC2 (5'CGCAGGATGTTACGCTTAACG3') to amplify a 426-bp nucleotide sequence within the carbamoylphosphate synthetase gene (48). DNA amplification was carried out in a 25 µl reaction consisting of 12 µl Gotaq® Green Mastermix (Promega Corporation), 3 µl template DNA, 1 µl each primers (total 4 µl), and 6 µl of nuclease free water. PCR was performed on Eppendorf thermal cycler (Eppendorf, Hauppauge, NY) with cycling conditions including an initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 sec, 60°C for 20 sec, 72°C for 30 sec, and a final extension at 72°C for 3 min. Amplified products were run on 1.5% gel made with 1x TAE buffer and electrophoresis run for a total of 1 hr. A total of 3 replications of the entire experiment were completed.

Confocal laser scanning microscopy (CLSM). To locate the inoculated pathogens on the fruit rind, a 1 cm^2 rind piece, out of 4 cm^2 of the inoculated square, was

divided into two pieces (0.5 cm^2 each) for analysis by scanning electron microscopy (SEM) and CLSM. Although all samples were collected and processed for both types of microscopy, for the latter, a total of 36 samples (one for each pathogen and control treatment and DPI in a single replication) were processed. Tissues were fixed in 4% paraformaldehyde for 1 h and washed 3X in distilled water. Fixed pieces were longitudinally hand sectioned with a razor blade and placed onto a glass slide with a drop of water and covered with coverslip. To visualize green fluorescence (GFPuv)- or red fluorescence (DsRed) - expressing *Erwinia* and *Salmonella*, respectively, sections were observed using a LEICA (Japan) TCS SP2 Laser Scanning Confocal Microscope with an upright Leica DMRE microscope, equipped with an Argon ion laser at 458, 476, 488 and 514 nm; green HeNe at 543 nm; and red HeNe at 633 nm; the Coherent UV Laser was at 300-360 nm. GFPuv was found to excite with 488 nm light and the emission was collected through a BA 505-525 filter. The wavelength of the lasers was first optimized using positive control samples inoculated with both pathogens, before processing the experimental samples.

Scanning electron microscopy (SEM). The remaining 0.5 cm^2 of the rind pieces of fruits sampled at 0, 9, or 24 DPI were processed for SEM. Tissues were fixed with 2% gluteraldehyde in 0.2 M cacodylate buffer and stored at room temperature for 2 h, rinsed 3X with 0.1 M buffered wash (60 ml 0.2 M cacodylate buffer and 12.3 g sucrose dissolved in 140 ml of dH_2O) and then fixed for 1 h in 1% osmium tetraoxide at room temperature. After another rinse they were dehydrated in ethanol [(30%, 50%, 70%, 80%, 90%, 95%, and 100% (3 X)] followed by critical-point drying 2X with HMDS (hexamethyldisilazane) and sputter coating with Au/Pd for 2 min with a MED 010

sputtering device (Balzers Union, Blazers, Liechtenstein). Coated samples were examined at different magnifications with a Quanta 600F scanning electron microscope (FEI Corporation, Hillsboro, Oregon), operating at 15 to 20 kV.

Statistical analysis. All experiments, including fruits inoculated with *E. tracheiphila* only, *S. enterica* only, a mixture of the two microbes, or 0.1% peptone water as a control, were completed in triplicate. Mean and standard errors of log base 10 transformed colony counts of both bacteria were calculated using MS Excel and the resulting data were analyzed using ANOVA procedures with SAS Version 9.2 (SAS Institute, Cary, NC). Main effect means (DPI given treatment and treatment given DPI) were reported and analyzed with planned contrasts. Percent data were analyzed with contingency tables and Fisher's Exact Test for the fruit detection part of the text. Graphs were plotted using SigmaPlot 2002 for Windows Version 8.0 (SPSS Inc.). All tests are considered significant at the $P \leq 0.05$.

Results

Fruit appearance and symptom development. Newly formed, healthy cantaloupe fruit were hairy and smooth-skinned, but at about 10-12 days of age, small cracks appeared in the rind around the blossom end. Red-to-orange exudates seeping from the newly formed rind cracks indicated the presence of a connection from the fruit interior to the outside environment (Figure III-3). The cracks lengthened, branched and intersected over time, gradually filled in and became raised as corky layers built up along them.

After *E. tracheiphila* or *E. tracheiphila* + *S. enterica* inoculation of cantaloupe fruit rind, small watersoaked lesions (Figure III-4 B) appeared at the inoculated site within 4 - 7 days on 28 (58%) of the inoculated fruit. 61% of the fruits, sampled at 24 DPI with *E. tracheiphila* alone treatment, had watersoaked lesion. The watersoaked spots ranged from barely noticeable lesions to a maximum of ca. 2 cm^2 (half the area of the inoculation site in a few fruits) over the next 20 days (data not shown). The percentage of fruit that developed watersoaked lesions by 9 and 24 DPI in these two treatments did not differ significantly ($P<0.05$) (Figure III-4 A). *E. tracheiphila,* tagged with GFPuv, was observed (using UV light) as patches of green fluorescence between the rind cracks and underneath the rind cuticle (Figure III-4 B and C). No lesions appeared on any fruits receiving *S. enterica* alone or the control buffer.

***S. enterica* and *E. tracheiphila* survival on cantaloupe fruit rind.**

Enumeration. After inoculation of *S. enterica, E. tracheiphila,* or a mixture of both species onto cantaloupe fruit rind, bacterial recovery varied with the sampling time. At 0 DPI, 3.62 log out of 5.60 log CFU/3 cm^2 inoculated bacteria were recovered in the *S. enterica*-only treatment and 3.69 log out of 5.63 log CFU/3 cm^2 in the *S. enterica* + *E. tracheiphila* inoculated treatment were recorded in Universal Pre-enrichment Broth (Table III-1). *S. enterica* numbers recovered in both treatments were significantly (<0.0001) lower (ca. 80% less) at 9 DPI than at 0 DPI, and by 24 DPI only 2 fruits (13%) receiving the *S. enterica* + *E. tracheiphila* treatment still had detectable numbers of *S. enterica* (Table III-1).

Unlike *S. enterica*, *E. tracheiphila* recovery was very low at 0 DPI. Only 1.58 log cfu/3cm^2 and 0.49 log cfu/3cm^2 were recovered from fruit treated with *E. tracheiphila*

alone, or with *E. tracheiphila* + *S. enterica*, respectively, out of 5.64 - 5.71 log cfu/3cm^2 inoculated (Table III-1). The latter recovery rate was approximately 70% less than that of the *E. tracheiphila*-only treatment. No *E. tracheiphila* was detected on fruits sampled at 9 and 24 DPI in either treatment when watersoaked lesions were not present.

Microscopy. At 0 DPI *S. enterica* was observed by CLSM on rind samples that had received both single and mixed culture inoculations (Figure III-5 A and B, respectively). Only a few samples were visually positive (on the surface) for *S. enterica* at 9 DPI, and none were positive at 24 DPI (data not shown). *E. tracheiphila* was observed on the rind surface at 0 DPI (Figure III-5 C), and at 9 and 24 DPI when watersoaked lesions were present (Table III-2). In internal longitudinal sections below the watersoaked lesions (to a depth of 3 mm), *E. tracheiphila* was observed in the intercellular spaces (Figure III-5 D).

S. enterica numbers on the rind surface at 0 and 9 DPI, as detected by CLSM observation and culture enumeration, were indistinguishable (data not shown). Although bacteria were not counted in CLSM, in some of the 0 DPI samples it was difficult to find *S. enterica*. The number of *S. enterica* varied within same DPI samples and between samples of 0 and 9 DPI, but this bacterium was never observed at 24 DPI. The number of *S. enterica* PCR positive fruits was significantly higher ($P>0.001$) at 0 DPI than at 24 DPI in both, single or multispecies inoculated samples (Table III-1, Figure III-6). *S. enterica* was detected (by overnight enrichment culture and PCR) on 14% and 40% of fruit inoculated with *S. enterica*, or with *S. enterica* + *E. tracheiphila*, respectively, at 24 DPI, but these treatments were not significantly different ($P = 0.11$, one tailed Fisher's Exact Test) (Table III-2, Figure III-6). Among mixed culture inoculated fruits sampled at 24

DPI, *S. enterica* survived on more fruits (50% - 4 out of 8) having *E. tracheiphila* -induced watersoaked lesions than on fruits without them (29% - 2 out of 7) (Table III-2).

Scanning electron micrographs of fruit having with watersoaked lesions, inoculated with *E. tracheiphila* or *E. tracheiphila* + *S. enterica*, revealed bacterial masses on the rind surface on or adjacent to the natural cracks (Figure.III-7 B) as well as deep inside the cracks (Figure.III-7 D). Few fruits at maturity with watersoaked lesions, ≤ 0.5 cm^2 lesion, showed inhibition of the watersoaked lesion with brownish margin and looked like drying out (data not shown).

***S. enterica* colonization of cantaloupe fruit sub-rind mesocarp.** Two types of mesocarp samples, one immediately underneath the *S. enterica* or *S. enterica* + *E. tracheiphila* inoculated rind and sampled at 0, 9 and 24 DPI (i.e. sub-rind mesocarp) and the other from the central core of the fruit that received no rind inoculations and was sampled only at 24 DPI (i.e. inner mesocarp), were examined. Neither microbial analysis (cultivation) nor PCR detected *S. enterica* in the sub-rind mesocarp of 112 fruits sampled in all DPI and treatments (Table III-3, some data not shown).

Assessment of systemic movement. Of the 131 total inner mesocarp samples, taken from the central core of fruits that received no inoculation but were growing on the same plants on which other fruit received either *S. enterica* or *S. enterica* + *E. tracheiphila*, and sampled at all DPIs, were negative for *S. enterica* by both microbial plating and PCR (Table III-2).

***E. tracheiphila* colonization of cantaloupe fruit sub-rind mesocarp.** Some of the sub-rind mesocarp of fruits that were inoculated with *E. tracheiphila* or *E.*

tracheiphila + *S. enterica*, and that later developed watersoaked lesions (sampled at 9 DPI and later), were positive for *E. tracheiphila* by microscopy, culture and PCR. On *E. tracheiphila* only inoculated fruit, *E. tracheiphila* was detected in 10% and 31% of sub-rind mesocarp sampled at 9 and 24 DPI, respectively (Table III-3). At 24 DPI, 27% of the sub-rind samples that received *E. tracheiphila* + *S. enterica* and had watersoaked lesions were positive for *E. tracheiphila*. All control fruits and those which did not develop watersoaked lesions were negative for both the pathogens on sampled sub-rind mesocarp.

Discussion

Outbreaks of foodborne illness associated with *Salmonella enterica* contaminated cantaloupe fruits underscore the importance of understanding the mechanisms of microbial contamination and persistence in the fruit. Recent work by others has shown that the presence of other microbial species, including plant pathogens, on the surfaces of a number of plant species can enhance rates of human pathogen survival and internalization. In this study we investigated the fate of *Salmonella enterica* Poona, alone or in the presence of the cucurbit wilt causing bacterium, *Erwinia tracheiphila,* on cantaloupe fruit surfaces.

In nature, striped and spotted cucumber beetles transmit *E. tracheiphila* while feeding on plant parts (13), and even frass can be a source of contaminating bacteria as beetles feed on flowers and released bacteria enter plant interiors and cause wilt symptoms (48). The formation of tears or cracks during progressive changes in shape and size of cantaloupe fruit (18) and their subsequent coverage by the accumulation of a

corky scar, are unique features that expose modified lenticels that serve in gas exchange (70). Smooth surfaced melons also develop corky ridges if exposed to mechanical injuries (40). Prior to the wound healing, however, the cracks may provide a ready pathway for microbes on the surface to enter interior tissues.

After rind inoculation, alone or in a mixture with *E. tracheiphila*, *S. enterica* could be detected on cantaloupe rind surfaces throughout the experiment, but its population levels declined over the successive sampling periods (0, 9 and 24 DPI) irrespective of the treatments. Others have shown that *S. enterica* can remain viable on the *Arabidopsis thaliana*, lettuce, parsley, radish, and carrot phyllosphere for an extended time (16, 36, 37). That *S. enterica* populations decline over time on agricultural produce also has been reported elsewhere and is not surprising, as many factors determine bacterial survival and the plant environment is generally not considered to be a natural niche for human enteric pathogens (4, 5, 9, 41). Although most fruit receiving *S. enterica* in our experiments tested positive only after enrichment, two fruit inoculated with the *S. enterica* + *E. tracheiphila* mixture still had countable *S. enterica* through direct plating at fruit maturity (24 DPI).

We found no evidence for invasion or colonization of the fruit mesocarp (sub-rind or inner mesocarp) by *S. enterica*. Human enteric pathogens are documented plant invaders under some conditions, having been reported to traverse lettuce stomata (42), and to colonize tomato leaf trichomes (4), roots (32) and flowers (31). Infiltration into cantaloupe fruit during low temperature storage (59) also has been found. The fact that we never detected *S. enterica* in any mesocarp samples during our study suggest that

even in the presence of watersoaking this bacterium rarely, if ever, traverses the rind into the edible portions of the fruit.

E. tracheiphila, which causes wilt in cantaloupe and many other cucurbit crops in the eastern United States (12, 19, 24), is transmitted in nature by spotted and striped cucumber beetles (29). Our original reason for including this treatment was the hope that this plant pathogen might serve as a positive control so that, if *S. enterica* were not detected on the cantaloupe rind or in interior tissues, we would know that the reason was not a failure of our inoculation method. Introduction of this bacterium, in volumes and titers unlikely to occur in the environment, directly onto cantaloupe rind surfaces, is far from a natural phenomenon. However, our preliminary experiments had revealed that *E. tracheiphila* could enter the fruit after introduction to the cracked areas (data not shown) or through flower interiors and produce watersoaked lesions (28). Furthermore, Rojas and Gleason (61) recently reported that *E. tracheiphila* can live as an epiphyte on muskmelon leaves under a wide range of leaf wetness levels and temperatures, and they speculated that this niche could serve as a source of *E. tracheiphila* inoculum for pathogen dissemination. Their findings, combined with ours, suggest that *E. tracheiphila* may be a normal resident on cucurbit plant surfaces in nature. If this is true, then its ability to facilitate the survival of a human pathogen such as *S. enterica* becomes much more than an academic question.

E. tracheiphila was detected on fruit rind soon after inoculation at 0 DPI, but only in very low numbers, and it was never detected from the surfaces of healthy looking fruit at 9 and 24 DPI. These low recovery rates for *E. tracheiphila* even at 0 DPI may be due to the slow growth rate of this species, high viscosity of bacterium with significant

amount of polysaccharide production, the unusual plant niche for this bacterium, or rapid loss in viability (13, 65, 66). Considerable research has been done to find the accurate inoculation (33, 51, 54, 57, 66, 73, 74), isolation (54, 66), and storage (13, 22) techniques for *E. tracheiphila*. Numerous methods of *E. tracheiphila* transmission in cucurbit plant has been studied (56) but its internalization through cracks formed on fruit surface has never been reported. However in our experiment, *E. tracheiphila* did traverse the rind, of some fruit leading to the formation of watersoaked lesions that enlarged over time. We detected *E. tracheiphila* in 31% of sub-rind mesocarp samples, that had lesions at 24 DPI, and the increase in their numbers in that location from 9 DPI to 24 DPI suggests that they either continue to move there over time or multiply there. That *E. tracheiphila,* deposited artificially in high numbers on the cantaloupe rind, can colonize the rind surface, enter the underlying mesocarp tissue through natural cracks, and cause watersoaked lesions is a new finding. Such events might take place in nature, but be un-noticed if contaminated beetles feed on these fruits or their frass contaminates the open wound as natural cracks on fruit surface.

Introducing the human pathogen, *S. enterica,* and the plant pathogen, *E. tracheiphila,* simultaneously led to some differences in the behavior of the individual bacterial species. In this work, *S. enterica* persisted in greater numbers in the presence of watersoaked lesions caused by *E. tracheiphila* than on non-symptomatic rinds. In nature, human pathogens that come into contact with potential plant niches encounter numerous microflora with which they may interact synergistically or antagonistically (8, 23, 38, 62). Microbial synergism between *S. enterica* and normal plant microflora, such as certain storage fungi (71), and the plant pathogen *Xanthomonas campestris* pv.

vesicatoria, in the absence of plant disease (5), has been reported. Recently, Barak and Schroeder (6), showed a positive correlation between bacterial speck lesion formation and *S. enterica* survival on the tomato phyllosphere. Our test pathogens, i.e. *S. enterica* and *E. tracheiphila*, might interact and colonize differently on other varieties of cantaloupe fruit; a study with *S. enterica* and *Escherichia coli* O157:H7 showed variable levels of root colonization depending on the cantaloupe variety (21). Both *S. enterica* and *E. coli*, colonized the rhizosphere of 'Burpee's Ambrosia' most and 'Israel Old Original' least among five cultivars tested. In the work reported here, it is likely that the leakage of cellular contents into intercellular spaces after *E. tracheiphila* inoculation, which resulted in watersoaking, provided nutrients and water supportive of *S. enterica* growth on the rind surface, thereby extending the persistence of the human pathogen in what would otherwise have been a less favorable environment.

We saw no indication that the presence of *S. enterica* influenced the behavior or survival of *E. trachiphila* on the cantaloupe fruit. The apparent lack of interaction between these species on rind surfaces is interesting because, in vitro, when *S. enterica* and *E. tracheiphila* are streaked onto the same agar plate, there is clear inhibition of *E. tracheiphila* (data not shown).

In this work there was no evidence for systemic movement of either pathogen in the cantaloupe plant after rind inoculation. Lack of systemic movement of *S. enterica* was expected, since we saw no internalization of this species. More interesting is that *E. tracheiphila,* which was detected in the fruit mesocarp and which, in "typical" wilt disease, moves systemically in the xylem, was not detected in un-inoculated fruit present on the same plant that had inoculated fruit. The question of whether *E. tracheiphila,* after

traversing the fruit rind into the mesocarp, can find its way to the xylem and from there move to other plant parts needs further investigation. In a preliminary experiment we found evidence for systemic movement of *E. tracheiphila* to the fruit then to the vines, resulting in plant wilting after flower interior inoculation (28). Twenty four days may not be enough time for the plant pathogen to move through the vines and cause wilting. Changes in fruit physiology during ripening, or the density of fruit tissues may restrict systemic bacterial spread. The fact that *E. tracheiphila* numbers declined over time may also reflect physiological incompatibility. Many storage and pathogenic fungi are active on mature fruit from where they initiate postharvest decay (67, 75), but there are only few bacterial diseases associated with fruits [*Erwinia amylovora* (26), *Xanthomonas axonopodis* pv. *citri* (43, 44), *Xyllela fastidiosa* (14), etc.], and their primary location is organs or tissues rather than fruits (39, 50).

Our results support the conclusion that survival of *S. enterica* on cantaloupe fruit can be influenced by synergism with other microflora. As *E. tracheiphila* did internalize in our study through the natural cracks, producing watersoaked lesions, the possibility of *S. enterica* internalization also exists. Fruit cracking may be a route of internal contamination in the field.

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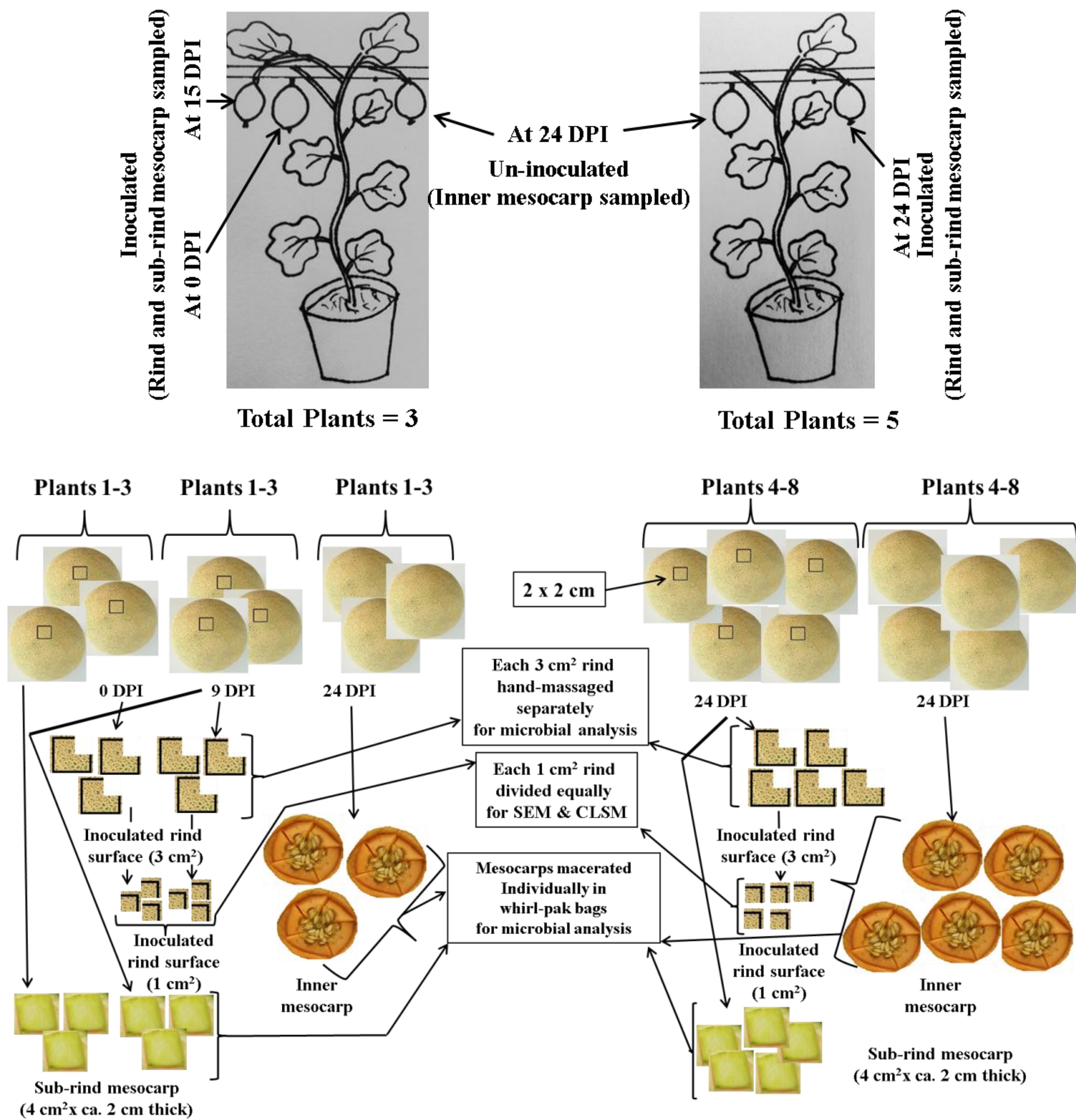

Figure III-1: Fruit sampling in a single replication. Fruits of 8 plants were inoculated with each of the three pathogen treatments (*E. tracheiphila* or *S. enterica* or mixture of these two pathogens) (24 plants), and three plants served as controls (24+3=27 plants per replication). Fruits of three plants per treatment (9 plants) were sampled at 0 and 9 DPI, and fruits of five plants per treatment (15 plants) were sampled at 24 DPI. Rind, sub-rind mesocarp and inner mesocarp were sampled as illustrated.

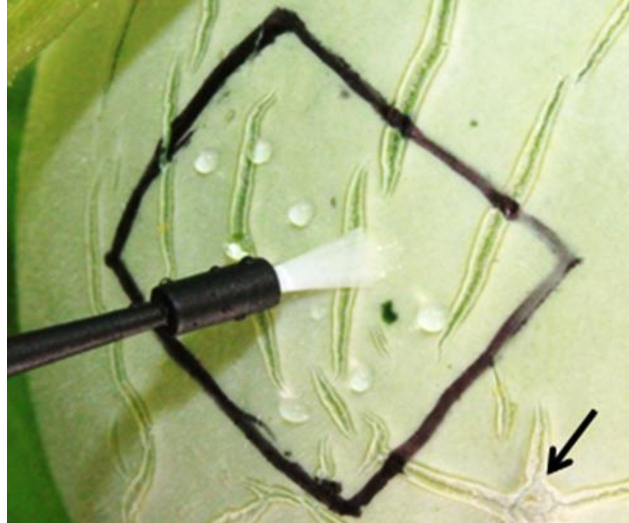

Figure III-2: Newly formed natural cracks on cantaloupe fruit rind areas (2 x 2 cm) inoculated with in 10-15 droplets of 20 μl pathogen(s) suspensions or 0.1% peptone and spread with a sterile bristled brush. Arrow head indicates cracks older than those in the area being inoculated.

Figure III-3: Reddish orange exudate (arrow) observed on the natural cracks of 10-12 day-old cantaloupe fruit rind. These cracks are naturally healed by deposition of corky material, forming the characteristic netting on cantaloupes.

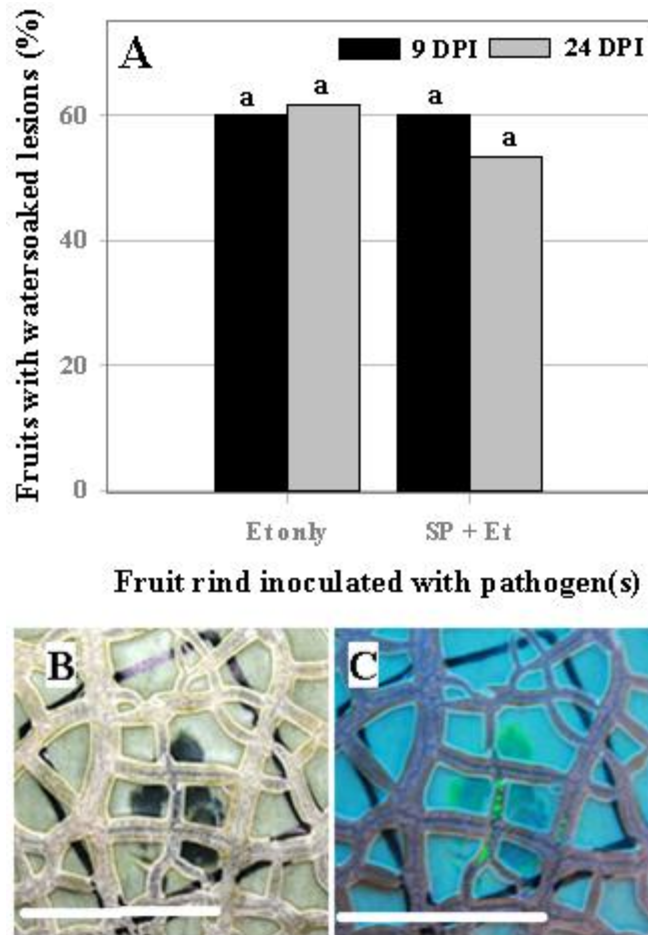

Figure III-4: Cantaloupe rind inoculation with *E. tracheiphila* (*Et*- green fluorescing with GFPuv), alone or together with *S. enterica* (*S*P- red fluorescing with DsRedExpress), pathogens were spread onto the rind surface with a soft brush and sampled at 0, 9 or 24 DPI. (A) Percentage of cantaloupe fruit showing watersoaked symptoms based on visual inspection, no significant difference between day 9 and 24 at any level of *Et* or *S*P + *Et* (Fisher's Exact one-tailed P=0.64 and P=0.53, respectively), (B and C) Cantaloupe rind with watersoaked lesion observed under natural light, and under UV light, respectively. C shows green fluorescing *E. tracheiphila* on the cracks and beneath the cuticle in a watersoaked area. Scale bars represent 2 cm.

Table III-1: Recovery of *S. enterica* Poona and *E. tracheiphila*, at intervals following inoculation, singly or together, onto cantaloupe fruit rind surfaces.

Treatments	Pathogen assessed	Development of watersoaked lesion	Pathogen recovery (Log CFU ± SE/3cm^2)		
			0 DPI	9 DPI	24 DPI
Et	*Et*	+	NA	TNTC (6/10)[¥]	TNTC (8/13)
		-	1.58^a ± 0.30 (8/10)	0.00^b ± 0.00 (4/10)*	0.00^b ± 0.00 (5/13)
*S*P	*S*P	NA	3.62^a ± 0.19 (9/9)	0.65^b ± 0.27 (4/9)	0.00^c ± 0.00 (0/14)
*S*P + *Et*	*Et*	+	NA	TNTC (6/10)	TNTC (8/15)
		-	0.49^a ± 0.34 (2/10)	0.00^b ± 0.00 (4/10)	0.00^b ± 0.00 (0/15)
	*S*P	NA	3.69^a ± 0.19 (8/10)	0.79^b ± 0.28 (5/10)	0.27^c ± 0.19 (2/15)
Control	*S*P and *Et*	NA	0.00 ± 0.00 (0, 3)	0.00 ± 0.00 (0/3)	0.00 ± 0.00 (0/6)

SE - standard error of mean, CFU – colony forming units, *S*P – *Salmonella enterica* Poona, *Et* – *Erwinia tracheiphila*
TNTC- Too numerous to count, NA- Not applicable since watersoaking did not occur immediately.
¥ Numbers in parenthesis following "TNTC" indicate # of fruits on which lesions developed /total fruit sampled for that treatment
* Numbers in parenthesis following pathogen recovery figures indicate # of fruit samples having detectable level of pathogen/total # fruit sampled.
Means within the same treatment having the same letter are not statistically significant at the 0.05 level according to ANOVA.

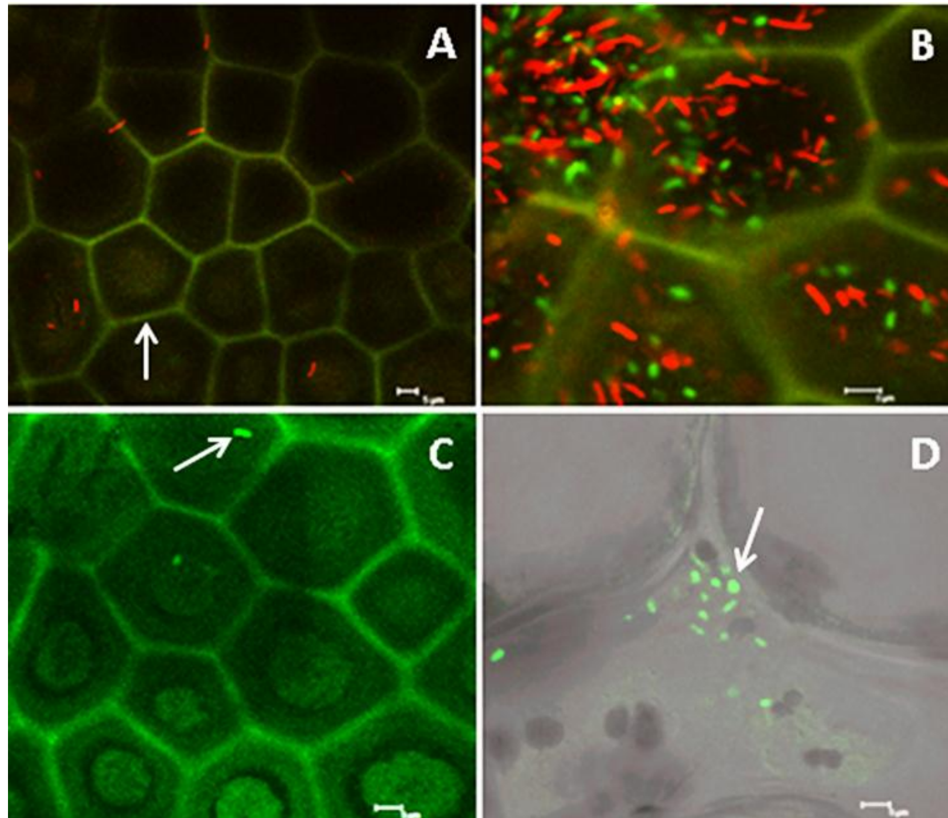

Figure III-5: Confocal laser scanning microscope images showing the presence of inoculated, fluorescently tagged bacteria on cantaloupe rind surfaces. Rind epidermal cells appear as a beehive pattern, and bacteria are indicated with arrows (panels C and D). (A) Fruit rind surface inoculated with *S. enterica* Poona (labeled with DsRedExpress) and sampled at 0 day post inoculation (DPI), (B) Fruit rind surface inoculated with a mixture of *S. enterica* Poona + *E. tracheiphila* (labeled with GFPuv) and sampled at 0 DPI (C) Fruit rind surface inoculated with *E. tracheiphila* and sampled at 0 DPI and (D) Longitudinal section of rind containing watersoaked lesion and sampled at 24 DPI; *E. tracheiphila* in the intercellular spaces (arrow) (inoculated with mixture of *S. enterica* plus *E. tracheiphila*). The scale bars represent 5µm.

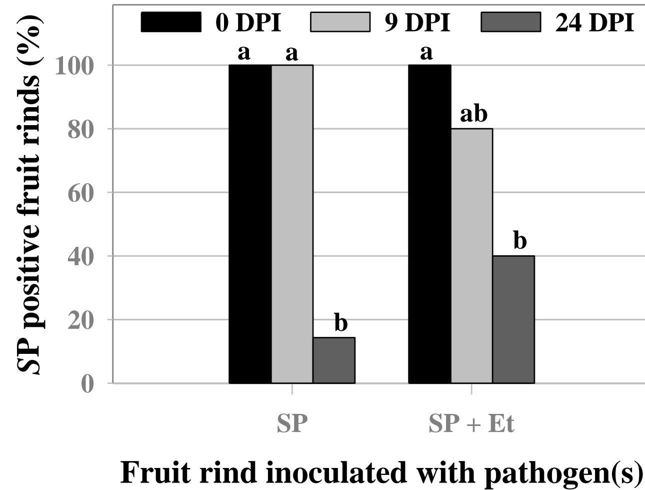

Figure III-6: Fruits positive for *S. enterica* Poona (*S*P) from rinds of cantaloupe inoculated with *S. enterica* Poona alone (*S*P), or *S. enterica* + *E. tracheiphila* (*S*P + *Et*), at 0, 9 or 24 days post inoculation (DPI). Similar letters above bars of the same treatment do not significantly differ at p < 0.05) according Fischer's Exact test- one tailed. Overall p-value for comparison of proportions among levels of DPI given treatments are <0.001 and 0.0039 for *S*P and *S*P + *Et*, respectively.

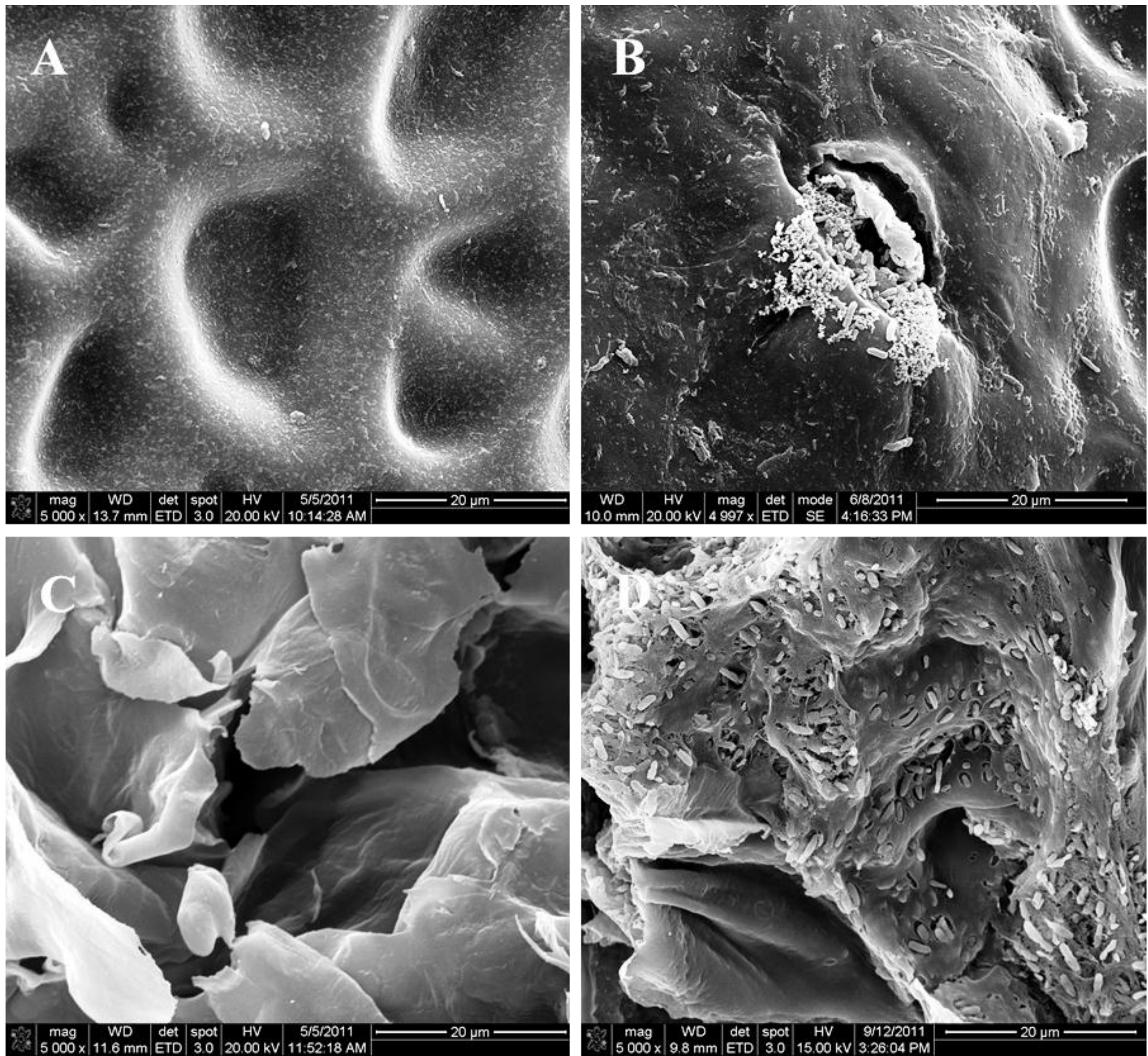

Figure III-7: Scanning electron micrographs of cantaloupe rind surface at fruit maturity (24 days post inoculation). (A) Rind inoculated with 0.1% peptone; (B) Masses of bacteria seen near a trichome scar on a rind that had a watersoaked lesion, inoculated with *E. tracheiphila*; (C) Crack on rind inoculated with 0.1% peptone; and (D) Crack on rind inoculated with mixed *S. enterica* + *E. tracheiphila* line the fruit crack that had a waterloaked lesion. All observations were made at 5,000X; scale bar shows 20µm.

Table III-2: Recovery of *S. enterica* from cantaloupe fruit inoculated with a mixture of *S. enterica* and *E. tracheiphila,* data sorted by the development of *E. tracheiphila*-incited watersoaked lesions.

Days post-inoculation	% of fruits with lesions + for *S. enterica*	% of fruits without lesions + for *S. enterica*	P- value
9	83.3 (5/6)*	75.0 (3/4)	0.67
24	50.0 (4/8)	28.5 (2/7)	0.38
Total	64.3 (9/14)	45.5 (5/11)	0.30

*Numbers in parenthesis following % pathogen recovery figures indicate # of fruit samples positive by (colony counts) of pathogen/total # of fruit sampled.

Percent fruit values analyzed using Fisher's Exact Test.

Table III-3: Percent of sub-rind mesocarp and inner mesocarp samples positive, by colony count and PCR, for *S. enterica* or *E. tracheiphila* from fruits inoculated with either *S. enterica, E. tracheiphila* or a mixture of these pathogens, sampled at different days post inoculation (DPI).

Samples for measurement	Treatment on fruit rind			
	Et	*S*P	*Et* + *S*P	Control
9 DPI				
Total # of fruits sampled	10	9	10	3
Sub-rind mesocarp*				
Et +	10[a]	0[a]	0[a]	0[a]
*S*P +	0[a]	0[a]	0[a]	0[a]
24 DPI				
Total # of fruits sampled	13	14	15	6
Sub-rind mesocarp				
Et +	31[a]	0[b]	27[ab]	0[b]
*S*P +	0[a]	0[a]	0[a]	0[a]
Inner mesocarp*				
Total # of fruits sampled	33	38	43	17
Et +	0	0	0	0
*S*P +	0	0	0	0

Et – *E. tracheiphila*, *S*P – *S. enterica* Poona,

***Sub-rind mesocarp**- 7-10 g of mesocarp underlying the rind square of fruits inoculated with pathogen(s) or with 0.1% peptone water.

****Inner mesocarp**- Mesocarp excised from the center of un-inoculated fruits on the same plants on which other fruit were treated with pathogen(s) or 0.1% peptone water treatment.

Percentages in the same row with the same letter are not significantly different at the 0.05 level. Means were separated using Fisher's Exact Test.

CHAPTER IV

SALMONELLA ENTERICA COLONIZATION OF CANTALOUPE FLOWERS AND FRUIT FOLLOWING FLOWER INOCULATION ALONE OR WITH *ERWINIA TRACHEIPHILA*

Abstract

Cantaloupe, which is vulnerable to *Salmonella* contamination, has been implicated in numerous outbreaks of foodborne illness. However, little is known about the mechanisms and pathways by which *S. enterica* colonizes the fruit. We hypothesized that bacteria present within flower interiors, to which they could be introduced by insects, could access the developing fruit through natural flower openings, such as nectaries and stigmas. We further hypothesized that the presence of a plant pathogen, the cucurbit wilt bacterium *Erwinia tracheiphila*, could influence the fate of *Salmonella* in this environment. Hand pollinated cantaloupe flowers were inoculated at the bottom of the floral whorl with 5 µl (ca. 10^7 cfu/ml) of *S. enterica* Poona, a clinical isolate from 2001 cantaloupe outbreak, *E. tracheiphila* MCM1-1, a mixture of these two pathogens, or 0.1% peptone water as a control treatment. Fruit mesocarp samples (25 g) were excised at 15and 43 days post inoculation (DPI). Whole flowers were sampled immediately (0 DPI)

and at fruit maturity (43 DPI) (consisted of dried floral remnants plus a 1 x 1 x 0.1-0.2 cm of adjacent blossom-end rind). All flowers sampled at 0 and 43 DPI and inoculated with either *S. enterica* or *S. enterica* + *E. tracheiphila* were positive for *S. enterica*, and at 43 DPI the populations of *S. enterica* were significantly (P<0.05) higher than these at 0 DPI from 4.46 to 6.12 log cfu/ ml and 4.89 to 6.86 log cfu/ml, respectively. *E. tracheiphila* was not recovered from any of the samples, regardless of treatment at 43 DPI and no observations were made at 15 DPI. An interior mesocarp sample from just one fruit, whose flower was inoculated with *S. enterica* only and sampled at 15 DPI, was positive for *S. enterica*. Our data suggest that, following flower inoculation, internalization of *Salmonella* into cantaloupe mesocarp is a rare event. However, dried floral remnants and the blossom end on mature fruit could act as a reservoir for *Salmonella* if the pathogen were introduced to the site at the flowering stage.

Introduction

Salmonella enterica is the human pathogen most common by implicated infoodborne illnesses, and outbreaks have been increasingly linked with the consumption of fresh fruits and vegetables (21). One million infections, 19,533 hospitalizations, and 378 deaths occur annually in the United States (40). Associated food recalls have resulted in significant economic losses (2, 9, 15). The first reported multistate outbreak of salmonellosis in the United States, in 1990, which was attributed to the consumption of *S. enterica* Chester contaminated cantaloupe (*Cucumis melo* var. *reticulatus*), was reported to affect 256 people (36), but the actual number of people involved was likely higher,

since many cases go unreported (29). Salmonellosis can be fatal for infants and immunologically compromised people (7).

Human pathogens, such as *Salmonella,* can enter agricultural production fields through agricultural inputs such as irrigation water (16, 32, 34), soil or animal manure (5). Cantaloupe is particularly vulnerable because of its surface netting and uaual contact with soil surface, where human pathogens may be present. Long term survival of human pathogens in agricultural environments creates a risk for consumers of fresh produce (11, 12, 18, 25, 45). Those pathogens, once established as surface contaminants, are not easily washed away, even when sanitizers are used (1, 28, 42, 44). Moreover, injuries on plant surfaces can prolong human pathogen persistence, possibly due to leaking fluids or the creation of protected niches (22, 45). Internalization of human pathogens in plants, and enhancement of their colonization of plant surfaces in the presence of plant pathogens or other microflora, have been demonstrated in lettuce, tomato and other fresh vegetables (4, 5, 13, 20, 26, 39, 45).

We hypothesized that *S. enterica* can internalize into the edible portion (mesocarp) of cantaloupe fruit after introduction into the flower interior, either alone or together with the melon wilt pathogen, *Erwinia tracheiphila*, and that *S. enterica* can survive on inoculated flowers until the time of fruit maturity.

Materials and Methods

Bacterial strains, maintenance and inoculum preparation. *S. enterica* Poona, a clinical isolate from 2001 cantaloupe outbreak, (2), and *E. tracheiphila* strain MCM1-1, isolated originally from cantaloupe by B. Bruton, USDA-ARS, Lane, OK, and provided

by M. Gleason, Iowa State University, IA, were used in this study. Bacteria were stored in Luria Bertani (LB) broth with 25% glycerol at -80°C. To prepare inoculum, *S. enterica* and *E. tracheiphila* were grown for 24 h at 37°C and 28°C on LB and nutrient agar, respectively. Cells of both pathogens were harvested with a sterile loop and dispersed in 0.1% peptone. The final concentration of both pathogens, determined by optical density and dilution plating, was adjusted to ca. 2 x 10^7 cfu/ml. For mixed species inoculation, equal volumes of *S. enterica* and *E. tracheiphila* suspensions were mixed with 0.1% peptone water to a final concentration of ca. ca. 10^7 cfu/ml. Suspensions were used immediately after formulation and inoculation was completed in 1-2 h.

Plant management. Cantaloupe, cv. Sugarcube, seeds were sown about an inch deep in cells of polypropylene flats containing Redi-earth potting mix (Sun Gro, WA) and placed in a growth chamber (75°F, 60% humidity and 14/10 h day/night light). Seedlings that were 21 days old and at the 2-3 leaf stage were transplanted in to 4.2 gallon plastic pots containing Metromix-300 potting mix (Sun Gro, WA) supplemented with slow-release Osmocote fertilizer (19N, 6P and 12 K). Pots were then transferred to a polypropylene tray in the greenhouse, where day and night temperatures were set at 24°C and 18°C, respectively, with 14 h day/ 10 h night light.

A week after transplanting the vining plants were trailed up and tied onto a framework of polyvinyl chloride (PVC) pipes, and pots were watered every other day. Young fruit that formed also were supported on the PVC frame. The experiment, which included three replications, was started in August 2011 and completed in January 2012. The average temperature and humidity recorded inside the greenhouse were 23°C and 52%, respectively.

Flower pollination, inoculation and sampling. Hand pollination was performed by collecting pollen, using a fine artist's paint brush, from 1-2 staminate flowers and then dabbing it onto the stigmas of pistillate flowers of the same plant; on the day they opened. Flower whorls were inoculated with pathogen(s) or with 0.1% peptone immediately after pollination. Cultured cell suspensions, adjusted to ca. 10^7 cfu/ml (5µl) were introduced, using a thin pipette tip, to the base of the floral whorl.

Two types of flower samples were collected to determine pathogen(s) survival: at day 0 samples were fresh, moist flowers and at day 43 samples consisted of the dried floral remnants supplemented with a thin, 1x1x0.1-0.2 cm from the blossom-end rind to which they were attached (Figure.IV-1). Two additional sample types: internal mesocarp tissues of fruits, which developed after flower inoculation, and excised at 15 DPI and 43 DPI, and from fruits that were left un-inoculated to test for systemic pathogen movement. Fruits were visually inspected for symptoms prior to sampling and analysis.

We hypothesized that *S. enterica,* inoculated in the interior of pollinated flowers, might traverse into the fruit derived from the ovary of that flower through natural openings such as nectarthodes, and from there could access the vascular tissue and move systemically into other regions of the plant, such as another fruit. To test for systemic movement, one un-inoculated fruit was left on each test plant for testing at fruit maturity. Treatments consisted of S. *enterica* alone, *E. tracheiphila* alone, or a mixture of *S. enterica* + *E. tracheiphila*, with a total of 5 plants per treatment (2 plants for sampling at 0 and 15 DPI and 3 plants for sampling at 43 DPI). For control treatments (0.1% peptone water), 1 and 2 plants were sampled at 0 and 15 and 43 DPI, respectively. This is

explained clearly, with a flow diagram, in Figure IV-1. Each treatment was replicated three times.

Microbiological analyses. Flower samples, fresh or dried with attached blossom end rind ca. ≤ 1 g each, inoculated or not with pathogen(s), were collected in individual whirl-pak bags (7 oz., Nasco Co., IL) macerated with a rubber hammer in 10 ml of Universal Pre-enrichment Broth (UPB) (Becton, Dickinson and Company, MD), hand shaken for 1 minute and processed for microbial analysis. Mesocarp samples were excised aseptically by bisecting the fruits, cutting wedge-shaped triangles perpendicular to the bisection, and then slicing and lifting a thin (2-4 mm thick) mesocarp layer that included the core seeds (Figure IV-2). Mesocarp samples (ca. 25 g) in whirl-pak bags (55 oz. size, Nasco Co., IL) were weighed and macerated as above. UPB (225 ml) was added to the bags followed by hand shaking for 1 minute. Flower and mesocarp suspensions were plated on xylose lysine deoxycholate agar (XLD) plates (250 µl in quadruplicate plates and 100µl in duplicate plates) to recover *S. enterica*. The presence or absence of *E. tracheiphila* was assessed by PCR. Reported optimal growth temperatures of these two pathogens differ (37° C for *S. enterica* and 28° C for *E. tracheiphila*), but in a preliminary experiment we found no difference in *S. enterica* growth rates on LB broth at 28° C and 37° C, based on optical density (OD) at 600 nm (data not shown), so all the enriched samples were incubated at 28° C for 24 hrs. The remaining flower and mesocarp suspensions were incubated and processed for *S. enterica* detection following Food and Drug Administration Bacteriological Analytical Manual (FDA, BAM) protocols (14). Finally, 1 ml of overnight incubated mesocarp and flower suspensions (1 ml each) were

centrifuged at 5800 x *g* for 10 minutes and the pellets stored at -20°C until the DNA was extracted for PCR.

PCR detection of *S. enterica* and *E. tracheiphila*. DNA was extracted from the frozen pellets using a DNeasy Blood and Tissue Kit (QIAGEN Group, Austin, TX). Pathogen presence was assessed by a multiplex PCR using *Salmonella* specific primers (forward- 5' GTGAAATTATCGCCACGTTCG GGCAA 3' and reverse- 5' TCATCGCACCGTCAAAGGAACC 3') to amplify a 284-bp nucleotide sequence within the *invA* gene (35), and *E. tracheiphila* specific primers ETC1(5'GCACCAATTCCGCA GATCAAG3') and ETC2 (5'CGCAGGATGTTACGCTTAACG3') to amplify a 426-bp nucleotide sequence within the carbamoylphosphate synthetase gene (30). DNA amplification was carried out in a 25 µl reaction mix consisting of 12 µl Gotaq® Green Mastermix (Promega Corporation), 3 µl template DNA, , 1 µl each primers (total 4 µl), and6 µl of nuclease free water. PCR was performed on Eppendorf Thermal cycler (Eppendorf North America, NY) with cycling conditions including an initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 sec, 60°C for 20 sec, 72°C for 30 sec, and a final extension at 72°C for 3 min. Amplified products were run on 1.5% gel made with 1x TAE buffer and electrophoresis run for a total of 1 hr. A total of 3 replications of the entire experiment were completed.

Statistical analysis. All experiments were completed in triplicate. Mean and standard errors of log base 10 transformed count values were calculated using MS Excel and the resulting data were analyzed using ANOVA procedures with SAS Version 9.2 (SAS Institute, Cary, NC). A two-factor factorial (treatment and DPI) in a randomized complete block design was the assumed model.

Results

Fruit appearance after flower inoculation. After *S.* Poona, *E. tracheiphila,* a mixture of the two bacteria, or peptone water were used to inoculate flowers, the range in the fruit shape and size did not differ from those of controls. Flowers, that remained attached to the fruit towards fruit maturity, became dry and brittle as fruits developed. All fruits, irrespective of treatments, appeared healthy from beginning to fruit maturity.

Survival and growth of *S. enterica* and *E. tracheiphila* at flower inoculation sites. Whole flowers (when available) from each treatment were collected and processed for microbial content. *E. tracheiphila* populations were not enumerated, but multiplex PCR was performed on DNA extracted from overnight enriched cultures. Except at 0 DPI, all samples were negative for *E. tracheiphila.* Immediately after flower inoculation recovery, of *S. enterica* was ca. 5 log in both *S. enterica* and mixed-culture treatments (Table IV-1); *S. enterica* recovery was significantly higher ($P<0.05$) at 43 DPI than at 0 DPI in both *S. enterica*-containing treatments. *S. enterica* recovery at 43 DPI was 39% greater (6.12 log cfu/ml) in the *S. enterica* only treatment, and 45% greater (6.86 log cfu/ml) in the *S. enterica* + *E. tracheiphila* treatment than at 0 DPI (Table IV-1). Rates of *S. enterica* recovery did not differ between the two inoculation treatments at 0 or 43 DPI ($P>0.05$). The identity of *S. enterica* was confirmed with multiplex PCR in these two treatments.

***S. enterica* and *E. tracheiphila* internalization of fruit mesocarp.** The mesocarp tissue of fruits that developed from inoculated flowers was sampled at 15 and 43 DPI to assess pathogen internalization. Of 108 fruits, including controls, sampled, 15 received *S. enterica* alone or in a mixture with *E. tracheiphila*. The mesocarp of one of 6

fruits that developed after flower inoculation with *S. enterica* alone and sampled at 15 DPI was positive for *S. enterica* after overnight enrichment. All other mesocarp samples were negative for *S. enterica.* We did not detect *E. tracheiphila* from any fruit mesocarp throughout the experiment.

Discussion

Salmonella, a common human enteric pathogen, has been implicated as a contaminant of cantaloupe fruit (3, 8, 31) and can be transferred into the edible mesocarp at the time of cutting (42). Whether it can invade developing cantaloupe fruit in the field is not known, although in a recent study *S. enterica* failed to enter cantaloupe plant roots after soil inoculation (27). Recent reports of *Salmonella* internalization into edible parts of the other plant species after artificial inoculation, either alone or in the presence of a plant pathogen, has increased concern that, under certain conditions, it could occur in the field (4, 5, 19, 25, 26, 37).

As numerous insects visit flowers and could transmit human pathogens (24, 43), we were interested to know whether *S. enterica* can survive in inoculated flower interiors and/or enter the cantaloupe plant through natural floral openings, such as nectarthodes or stigmas, and also whether the presence of the cucurbit wilt pathogen, *E. tracheiphila,* would influence that ability. Under the conditions of our study, the incidence of fruit mesocarp colonization by *S. enterica* after flower inoculation was very low. *S. enterica* did, however, survive on inoculated flowers until fruit maturity. Although we did not detect, *E. tracheiphila*, at fruit maturity, it is possible that microbial community members could have enhanced its survival.

Whether it was inoculated alone or together with *E. tracheiphila*, *S. enterica* was not found in fruit mesocarps sampled 43 days after flower inoculation. One *S. enterica* only-inoculated fruit, sampled at 15 DPI, was PCR positive, but only after enrichment culture, suggesting that population levels were very low.

Most flowers, regardless of treatment, were wet the day after inoculation/ pollination, and this could be a normal phenomenon for plant to make conducive environment for fertilization, and the presence of a film of water is often conducive to bacterial entry (46). Barak et. al. (4) reported that the broken bases of type 1 trichomes present on tomato leaves as a preferred site for *S. enterica* Poona colonization and the occurrence of disease in those plants correlated with higher *S. enterica* populations compared to those on healthy plants. Others have reported internalization of *S. enterica* in other fresh produce (18-20, 26). Guo et al. (19) found that 25% of tomato fruits contained *S. enterica* after flowers were brushed with a bacterial suspension. The fact that *S. enterica* Poona was significantly more likely to internalize than four other *Salmonella* serovars tested suggests the existence of serovar-specific traits that may influence adaptation to the plant environment. Human pathogen internalization through other plant parts also has been reported. *S. enterica* entered lettuce leaves through stomata (26), tomato fruit through roots (20), and stems inoculations (19). Greater fruit colonization by *S. enterica* occurred when tomato stems were inoculated prior to, rather than after fruit set (19). Similarly, fruit internalization by microbes other than human pathogens through unusual routes also has been reported. *Pseudomonas corrugata* (41) and *E. carotovora* subsp. *carotorova* (6), respectively, entered tomato fruit after flowers were sprayed or fruits were dipped in the pathogen inoculum. *P. corrugata,* which causes tomato pith

necrosis, usually infects through the rhizosphere, so the flower is an unusual route of internalization for this pathogen. *E. carotovora* subsp. *carotovora,* which causes soft rot of fruits and vegetables, is an important disease in the field and during storage.

Much of the previous research done to explore the possibility of human pathogen internalization involved relatively high doses of pathogens that are unlikely to occur in agricultural environments. When we used ca. 5 x 10^4 cfu/flower (ca. 10^7 cfu/ml) of *S. enterica*, a titer that would be realistic for most microorganisms in natural environments (23, 33), the bacteria survived and grew in the inoculated flowers. Although greenhouse humidity was relatively low (52% on average, and occasionally as low as 10%) the flower interior is likely to retain moisture, and nectar could serve as a source of nutrition. All flowers receiving *S. enterica* inoculation sustained population increases evident at 43 DPI ($P<0.05$). It's the population growth was greater when it was co-inoculated with *E. tracheiphila* than when it was inoculated alone. However, the latter bacterium was detected neither on surfaces nor in samples at 43 DPI. The decline in population of *E. tracheiphila* in this study is consistent with that reported previously after cantaloupe phyllosphere inoculation (38), although in the latter study it internalized in the fruit mesocarp after flower inoculation, producing watersoaked lesions (17). As we did not sample flowers between 0 and 43 DPI, we do not know the pattern of bacterial multiplication in this period, but it is possible that *E. tracheiphila* modified the environment such that it was more conducive for *S. enterica* survival and multiplication. Our data are consistent with an interpretation of a synergistic relationship between this human pathogen and other microflora; similar to that been reported by others for certain phytobacteria (5) and storage and pathogenic fungi (10, 37, 45). We did not detect *E.*

tracheiphila on any of the flowers sampled at 43 DPI and only short-term survival of this pathogen, under optimal conditions, has been reported on the cantaloupe phyllosphere (38). Furthermore, we cannot say whether *S. enterica* survived on or in the blossom-end rind that was combined with the flower sample. However, our data suggest that the likelihood of *S. enterica* internalization in cantaloupe is low and might occur only in special conditions that are unlikely to occur in the field.

Our work provides new information about the possibility of long-term *Salmonella* survival on artificially inoculated blossoms, and internalization into the fruit after flower inoculation. Further research is needed to better characterize the relationships between *Salmonella* and members of the natural microbial community. Survival of *S. enterica* on flower blossom could be a problem of having cross contamination if the pathogen is brought to the flower.

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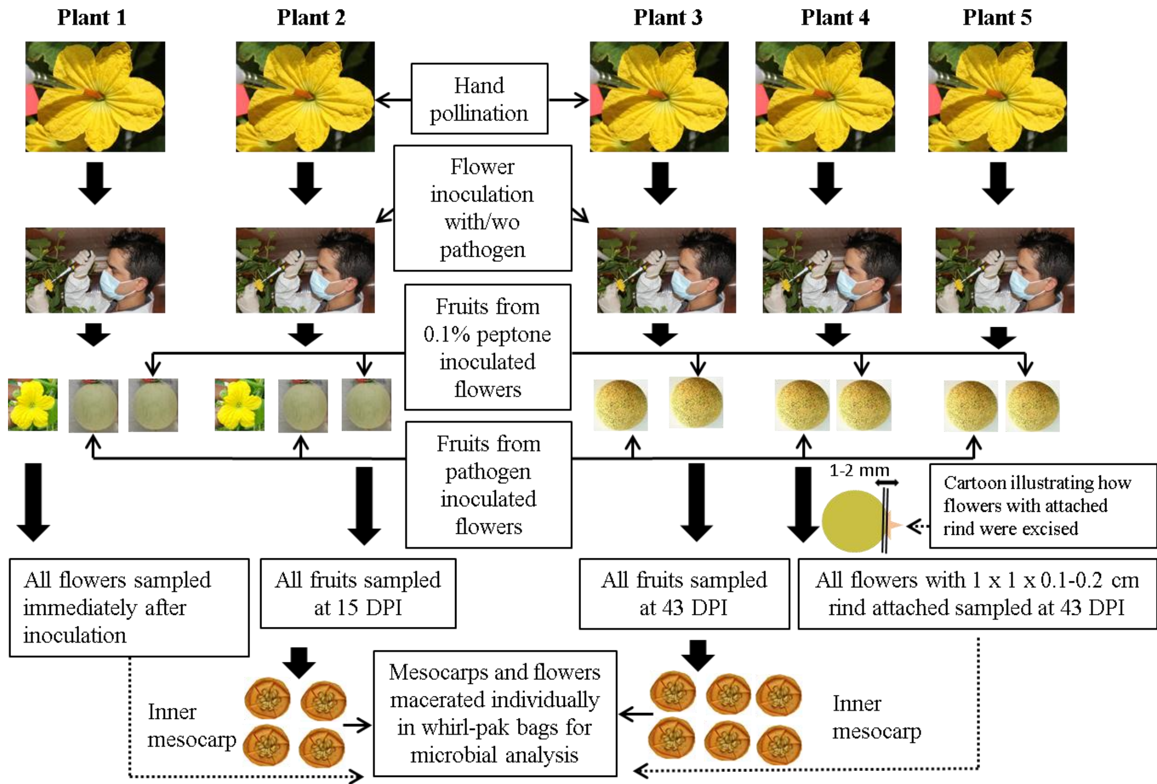

Figure IV-1: Illustration for fruit sampling from a single replication composed of 5 plants, flowers of which were inoculated with pathogens at the base of the floral whorl. Two plants each were sampled at 0 (for flowers) and 15 DPI (for inner mesocarp), and 3 plants receiving pathogen treatment *E. tracheiphila* alone, S. *enterica* alone, or a mixture of these two pathogens) were sampled at 43 DPI. Inner mesocarps and flowers (along with a small piece of attached rind) were sampled at 24 DPI. Control plants with 0.1% peptone inoculation were 1 and 2 for 0 and 15 DPI, and 43 DPI sampling, respectively, in each replication.

Figure IV-2: Fruit mesocarp samples excised at 43 DPI from fruits that developed from flowers previously inoculated with *S. enterica* or a mixed culture of *S. enterica* + *E. tracheiphila*. Each sample included edible mesocarp, seeds and placenta. Samples (ca. 25 g) were assayed by direct plating, enrichment and PCR techniques.

Table IV-1: Mean recovery of *S. enterica* Poona from flowers after inoculation with *S. enterica* alone or *S. enterica* + *E. tracheiphila* at 0 and 43 days post inoculation (DPI).

	Number of flower samples		*S. enterica* Poona recovery (Log_{10} cfu/ml¥)	
Treatment	0 DPI	43DPI	0 DPI	43 DPI*
*S*P only	15	10	$4.46^{b} \pm 0.03$	$6.12^{a} \pm 0.42$
*S*P + *Et*	15	11	$4.89^{b} \pm 0.02$	$6.86^{a} \pm 0.70$

*Values in a row followed by the same letter are not significantly different at 0.05 according to ANOVA.

*S*P- *S. enterica* Poona, *Et*- *E. tracheiphila*,

¥ Volume of wash water i.e. Universal Pre-enrichment Broth (UPB)

APPENDICES

Preliminary Experiments

Prior to beginning of the main experiments described in the preceding chapters, several preliminary studies were conducted to select an appropriate cantaloupe cultivar and to establish optimum inoculation and sample processing techniques.

APPENDIX A

Cantaloupe cultivar selection

Objectives. The objectives of this study were 1) to understand the general characteristics of cantaloupe plant growth, flowering and fruiting under the conditions of our BSL-2 greenhouse, and 2) to compare the plant habit, days to fruit maturity, number of fruits per plant, fruit weight, of three cantaloupe varieties, Sugarcube, Caravelle and Cruizer, and select the one most suitable for our purposes.

Greenhouse conditions. The average temperature recorded throughout this study was 23°C. Daylength was set at 14 h day/ 10 h night.

Plant form and growth. Although the leaves of most of varieties were bigger in the greenhouse compared to their natural size in the field, cv. Sugarcube was the most compact of the three tested, and therefore was the most amenable to vine trellising and

the easiest to keep vines of adjacent plants separate. Furthermore, fruits of cv. Sugarcube were the smallest of the three, and less likely to fall when hanging on the supported frame, as only peduncle were tied for fruit support. Therefore, cv. Sugarcube was selected for our study.

<u>Flowering</u>. All three cantaloupe varieties produced three types of flowers i.e. male (Fig. 1 A), complete (Fig. 1 B) and female (Fig. 1 C). Male flowers were produced early during plantgrowth. They were first observed 10-15 days after transplanting and continued to appear until plant death. Complete flowers, which appeared only after some male flowers were present, were the most likely to produce fruit. The time from planting to the appearance of the first complete flower ranged from 25-36 days (avg. 31) in Sugarcube, 37-44 days (avg. 41) in Caravelle and 36-38 days (avg. 37) in Cruiser. Female flowers were few in number, appeared near plant maturity, and seldom set fruit.

Pistillate flowers (mainly complete flowers) of cv. Sugarcube only were pollinated, using a fine artist’s paint brush, with pollen collected from 1-2 male flowers (Fig. 2 A- F). The pollen-laden brush was dabbed against the stigma tip several times for successful pollination. In Sugarcube, out of 32 pollinated flowers only 4 (13%) flowers produced fruits. Many small fruits aborted between 4-5 days, turning yellow and later shriveling.

<u>Fruiting and net formation</u>. Fruits, when newly formed (7-10 days of age), were light green in color and smooth surfaced but hairy. Rind cracking, a natural process resulting from fruit expansion that precedes the deposition of callose netting, began within 9-13 days of fruit formation. Cracks usually appeared first at the blossom end and

spread to cover the whole fruit within 10-15 days. Reddish or orange exudates observed in the crevices showed that the wound briefly exposed interior tissue (Fig. 3 A- C). Although we did not measure the crack depths, those of cv. Sugarcube were deep and more widely separated than those of cvs. Cruiser and Caravelle. The cracks later become filled with corky material that gradually build up to create the reticulation characteristic of mature fruits (Fig. 3 D- F).

The number of fruits set on each plant varied with cultivar. Average fruit numbers/plant in our experiments was 2-4 for Sugarcube, and 1-2 each for Caravelle and Cruiser. Days frompollination to fruit maturity ranged from 37-45 days (avg. 39), 29-40 days (avg. 36), and 38-42 days (avg. 40) for cvs. Sugarcube, Caravelle, and Cruiser, respectively. Overall, cv. Sugarcube matured the fastest, had the greatest fruit set and was most manageable in the greenhouse due to its compact form (Fig. 4).

APPENDIX B

Evaluation of pathogenicity on cantaloupe plants of a parental, and a GFPuv tagged derivative, of *E. tracheiphila*

Objectives.

1. To evaluate whether *E. tracehiphila* strain MCM1-1, which had been stored at -80°C for several years, was still pathogenic to cantaloupe, and to become familiar with symptoms produced on cantaloupe cv. Sugarcube.

This strain was collected originally from an Oklahoma cantaloupe plant by B. Bruton (USDA ARS, Lane, OK), and was obtained from M. Gleason (Iowa State University, Iowa). Stab inoculation was used to introduce the pathogen (10^7 cfu/ml @ 20 µl) onto the stem surface on the node of Sugarcube cantaloupe into the stem interior tissues of plant. All the inoculated plants became wilted,followed by shriveling of stem and then plant death..

2. To genetically modify *E. tracheiphila* strain of MCM1-1 to express the GFP gene, and to test its pathogenicity on cv. Sugarcube.

We tagged *E. tracheiphila* so that the pathogen could be traced by confocal laser scanning microscopy (CLSM) after fruit surface inoculation. An *E. tracheiphila* plasmid was transformed with pGFPuv (Clontech Laboratories, Inc., CA, USA) by electroporation as described in Ma et al. (1) and colonies were selected on ampicillin amended nutrient agar (NA-amp) plates. For inoculation, *E. tracheiphila* was harvested from NA-amp plate cultures after 24 hr. of incubation. In two sub-experiments, cantaloupe plants (cv. Sugarcube) were greenhouse grown to a height of 2-3 ft. and, 10-15 days after transplanting, were stab-inoculated (two spots per plant) with *E. tracheiphila* by depositing 20 µl on the stem surface. The inoculated surface was pricked at least 10 times with a syringe needle (Fig. 5 A) to create openings for bacterial entry. Plant shoots, above the site of inoculation, started to wilt 4-5 days after inoculation (Fig. 5 B). Stems then shriveled at the point of inoculation. Symptomatic stem pieces of 2 – 3 cm were surface sterilized with 1% sodium hypochlorite (NaOCl) for 2 minutes, aseptically excised, and plated on NA-amp (?) plates. Within 2-3 days of incubation at 28°C, colonies resembling *E. tracheiphila* (small colonies fluorescing green) appeared,

and 2-3 randomly picked colonies were confirmed to be *E. tracheiphila* with PCR. Colonies isolated from plants infected with GFPuv tagged *E. tracheiphila* appeared green under UV light (Fig. 5 C); colonies from these plants also were verified by PCR using *E. tracheiphila* specific primers (F- GGCGATCACGACACAGTTG T and R- CAGTTTTTGGTCAGGGCATA CTC) yielding a product of 68-bp, the expected size.

The *E. tracheiphila* strain, parental as well as GFP tagged, was still pathogenic, leading to wilting of the variety Sugarcube in our greenhouse condition.

APPENDIX C

Internalization of *Erwinia tracheiphila* into cantaloupe fruits through flower inoculation

To study the ability of human pathogenic *S. enterica* to survive on, and to enter and translocate, in cantaloupe plants, we needed to identify a positive control; i.e., a pathogen that was known to have the capability to do these same functions. However, the only serious bacterial pathogen of cantaloupe, the wilt-causing *E. tracheiphila,* is normally transmitted from plant to plant by insects (cucumber beetles) and we did not know what it would do when introduced onto natural cantaloupe rind cracks or into flowers.

Objective. The objective of this study was to evaluate whether *E. tracheiphila* was able to internalize into fruits after flower inoculation.

Methods. *E. tracheiphila* was inoculated in 5 µl volumes at concentrations of 10^7, 10^8, 10^9, and 10^{10} cfu/ml within the whorl of individual pistillate flowers. Concentration of *E. tracheiphila* was confirmed by OD_{600} nm and by plating on nutrient agar plates (NAP). Cultures were kept on ice until the time of inoculation, which was completed within 1-2 h after inoculum formulation. Fruits were visually inspected during their growth for symptoms. The presence of *E. tracheiphila* was confirmed by culturing fruit sample (after sterilization with 1% NaOCl for 1 min) in NAP and cultures were further verified by PCR using *E. tracheiphila* specific primers (F-GGCGATCACGACACAGTTG T and R- CAGTTTTTGGTC AGGGCATAC TC).

Results. Neither the cantaloupe plants nor the fruits developed any wilting symptoms. However, on one of 5 plants whose pistillate flowers were inoculated (10^9 cfu/ml) watersoaked lesions appeared on 2 out of 5 fruits (Fig. 6 A). Two out of three plants inoculated with *E. tracheiphila* at10^8 cfu/ml showed wilting symptoms, but no watersoaked lesions on fruits. The other *E. tracheiphila* inoculated plants, and all of the control plants, showed no wilting.

Plants that had fruits with watersoaked lesions also showed vine wilting (Fig. 6 B) and the peduncle that connected the vine and the fruit became shriveled and collapsed. Presence of *E. tracheiphila* was confirmed by the ‘ooze test’ (observation of a cloudy exudate emanating from a freshly cut stem, indicative of a slime-producing fungal or bacterial wilt pathogen) (Fig. 6 C). Fruits that developed lesions also had impaired netting (Fig. 6 D) and did not mature, and on some of them bacterial ooze also seeped out from the lesions. The interior tissue (mesocarp) of fruits showing watersoaked lesions were positive for the presence of inoculated bacteria by culturing (Fig. 6 E). On the other

hand, fruits that developed from un-inoculated plants (or flowers) appeared healthy and had no lesions (Fig. 6 F). PCR using *E. tracheiphila* specific primers (F-GGCGATCACGACACAGTTG T and R- CAGTTTTTGGTCAGGGCATAC TC) yielded a product of 68bp (the expected size) confirming the pathogen's presence inside fruits with lesions and wilted vines (Fig. 6 G).

Conclusion. *E. tracheiphila*, at high concentration (10^9-10^{10} cfu/ml) is able to traverse from the flower interior into the developing fruit, where it produces watersoaked symptoms. The bacteria also able moved into the vines after traversing through the fruits, where wilting occurred. In nature, only are the only known means for pathogen transmission is via the feeding of cucumber beetles. There is a possibility of *E. tracheiphila* internalization if insect frass falls within the flower whorl.

APPENDIX D

Internalization of *Erwinia tracheiphila* into cantaloupe fruits through rind cracking and its interaction with *Salmonella enterica*

Objective. Continuing to explore the use of *E. tracheiphila* as a positive control for *Salmonella* interactions with cantaloupe, an experiment was conducted to evaluate whether *E. tracheiphila* could internalize through natural cracks formed on the fruit and whether its presence affected *Salmonella* survival on those fruit surfaces.

Methods. Flowers on 8 plants were pollinated as described above and two fruits/plant were allowed to set. Fruits at the age of 10-12 days were inoculated with 10^7 cfu/ml of 20µl of *S. enterica* or a mixture of *S. enterica* + *E. tracheiphila*. The rind of

each fruit was marked with four 2x2 cm squares all of which were inoculated with *S. enterica* only, *S. enterica* + *E. tracheiphila* and 0.1% peptone water. Fruits were then sampled at 7 or at ca. days post inoculation 19 DPI (at maturity). A few of the fruits inoculated with *S. enterica* plus *E. tracheiphila* developed watersoaked lesions while fruits inoculated with *S. enterica* alone, or with peptone water (controls), were apparently healthy.

Results. At 7 DPI, *Salmonella* was detected on 100% (10 of 10) fruits previously inoculated with *S. enterica* alone or *S. enterica* plus *E. tracheiphila* (Table 1). At 19 DPI (fruit maturity) *S. enterica* survival varied with the treatments. Eighty percent of 5 fruits sampled and 86% of 7 fruits sampled had recoverable bacteria after previous inoculation with *S. enterica* only or a mixture of *S. enterica* + *E. tracheiphila,* respectively (Table 1).

Watersoaked lesions were observed on rinds co-inoculated with the two pathogens and the sub-rind mesocarp immediately below the sampled rind also were watersoaked (Fig. 7 A and B). Internal tissues (sub-rind mesocarp) of 2 fruits (out of 12) co-inoculated with *S. enterica* and*E. tracheiphila* on their rind surface showed *E. tracheiphila* lesions: one each at 7 DPI and at 19 DPI). Two fruit inner mesocarp yielded *Salmonella* on XLD plates (Fig. 7 C).and *E. tracheiphila* also was observed on ampicillin amended nutrient peptone agar on the same sample that was positive for *S. enterica* (Fig. 7 D). *Salmonella* identity was confirmed by PCR (using the *invA* primer pair). Fruits receiving *S. enterica*-only treatments and control plants were apparently healthy and rind and mesocarp samples from these plants were negative for both pathogens.

A total of 9 samples either (a) inoculated with *S. enterica* only, and sampled at 7 DPI (3 samples) or 19 DPI (1 sample) or (b) inoculated with a mixture of *S. enterica* plus

E. tracheiphila and sampled at 7 DPI (3 samples) or 19 DPI (1 sample) were processed for SEM observation. Biofilm like structures, bacterial cells seen as clustering together with some aggregated mass, were evident on samples inoculated with *S. enterica* only (Fig. 8 A and B), especially at 7 DPI. No biofilms were observed on samples receiving mixed culture (*S. enterica* + *E. tracheiphila)* inoculation (Fig. 8 C and D).

Discussion. *E. tracheiphila* may facilitate the internalization and surface survival of *S. enterica* inoculated onto the cantaloupe fruit rind at the time of natural fruit cracking. *E. tracheiphila* internalization into fruit and production of watersoaked lesions suggest that the cracks provide an opening into the fruit interior. The fact that *S. enterica* internalized into cantaloupe fruits only when co-inoculated with *E. tracheiphila* shows that the presence of *E. tracheiphila* may enhance the fitness and invasiveness of *S. enterica.* The biofilm like structures observed on the fruit surfaces, or in the cracks, inoculated with the mixed bacteria inoculum, shows that the two pathogens may interact with one another as well as with the host plant. Their ability to traverse into the sub-rind mesocarp has implications for our ability to remove the microbes with sanitizers, and re-emphasize the importance of good agricultural practices to maintain contamination free agricultural products to produce safe and healthy cantaloupe fruit.

LITERATURE CITED

1. **Ma, L., G. D. Zhang, and M. P. Doyle.** 2011. Green fluorescent protein labeling of *Listeria*, *Salmonella*, and *Escherichia coli* O157:H7 for safety-related studies. PLoS ONE **6**. e18083

Figure 1. Three types of flowers produced by cantaloupe plants A. Male flower, B. Complete flower (with male and female part) and C. Female flower.

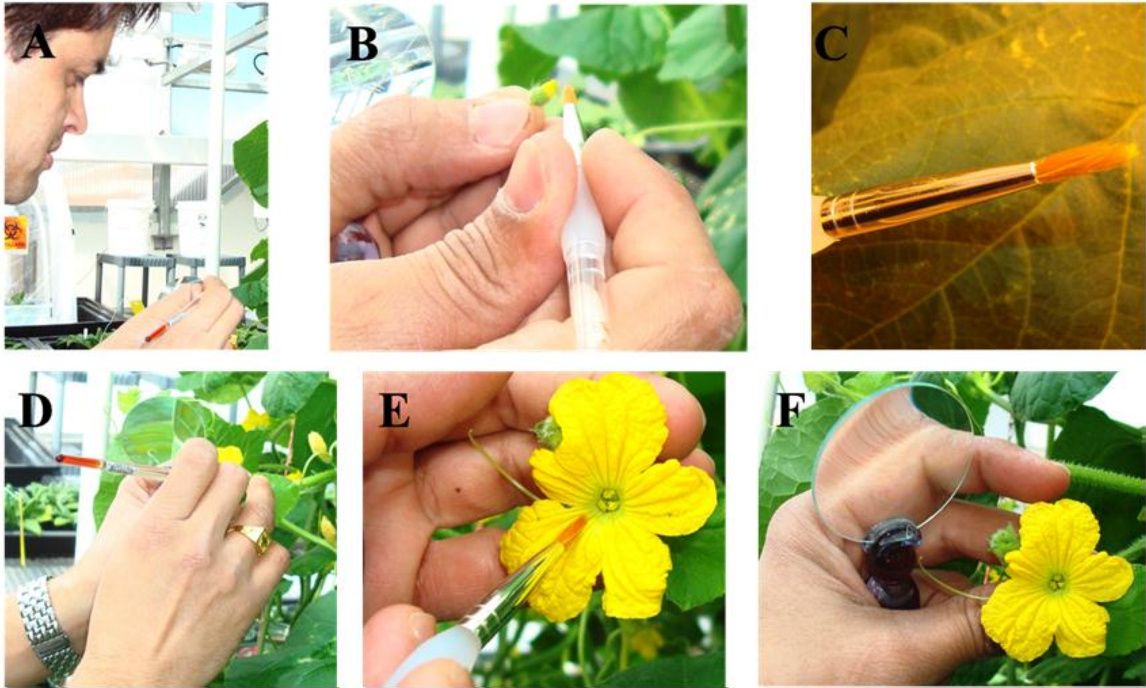

Figure 2. Hand pollination of cantaloupe flowers. A. Separating petals from the male flower to collect pollen; B. Collecting pollen with a fine artist's brush; C. Pollen collected on brush, ready for pollination; D. Brushing stigma of a complete flower; E. Complete flower after pollination; and F. Observing pollen adhering to stigma with a hand lens.

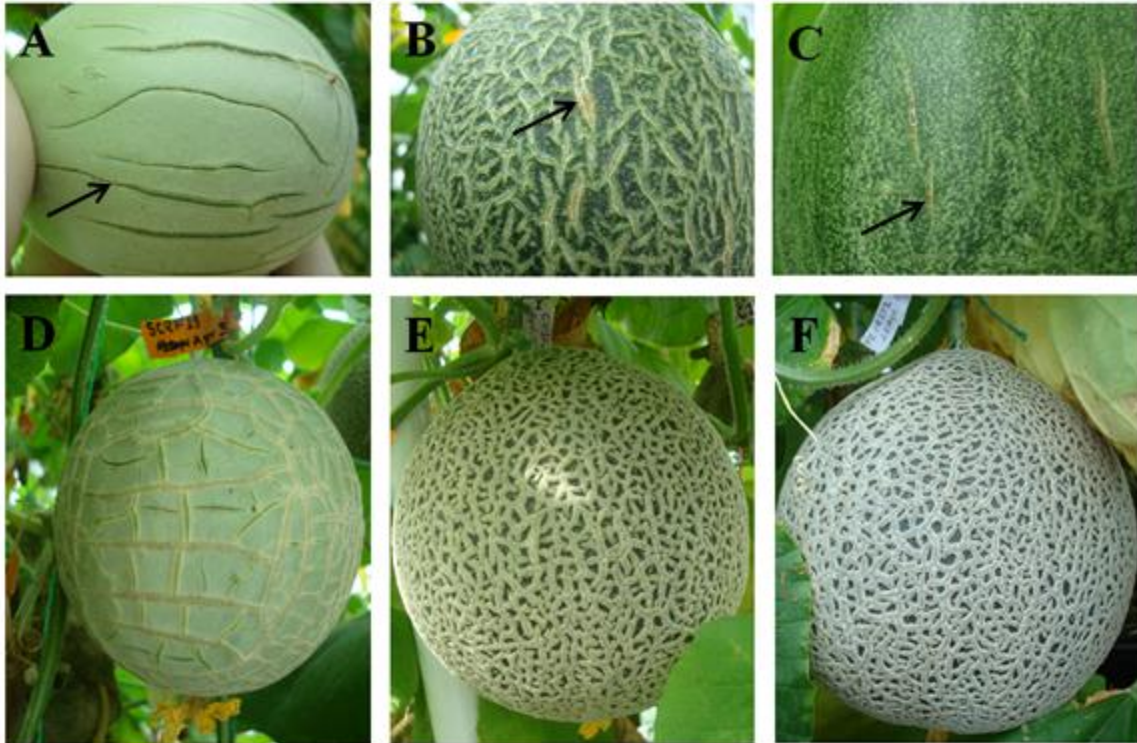

Figure 3. Cantaloupe varieties Sugarcube, Caravelle, and Cruizer at the time of cracking (A, B, C and D, respectively), during netting and towards fruit maturity (D, E, and F, respectively). Arrow head on pictures shows reddish orange exudates on cracks suggesting opening into the fruit.

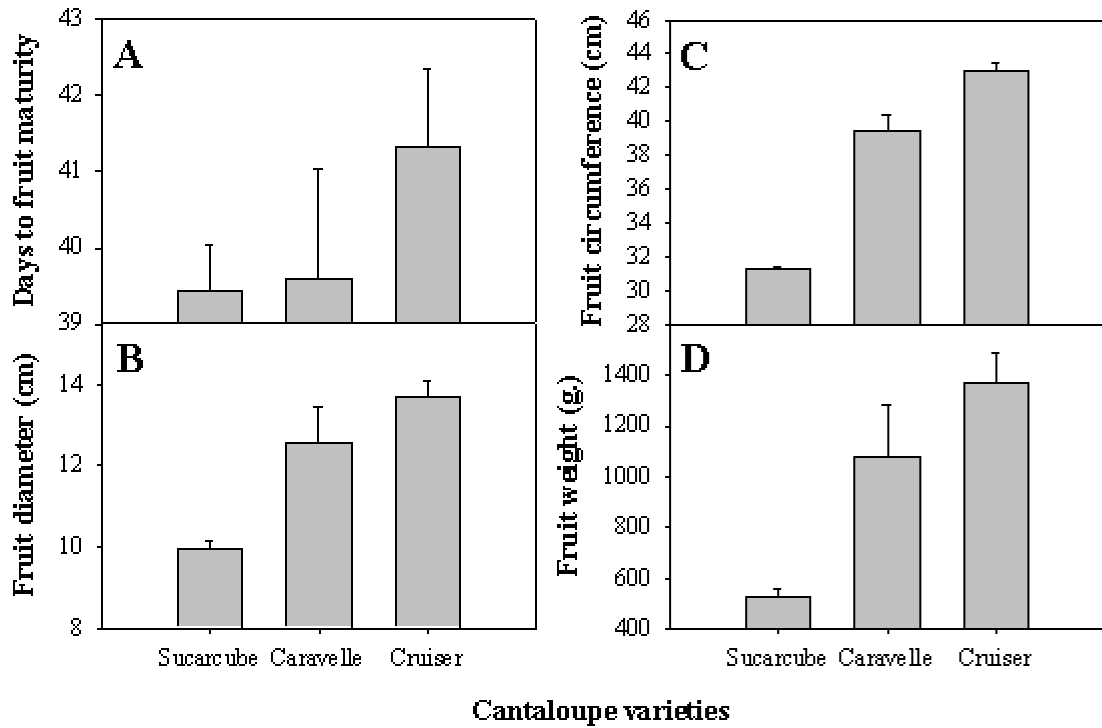

Figure 4. Reproductive parameters of three different cantaloupe varieties [Sugarcube (n=16), Cruiser (n=6) and Caravelle (n=5)] in the greenhouse during summer of 2010. The bars show the standard error for each category.

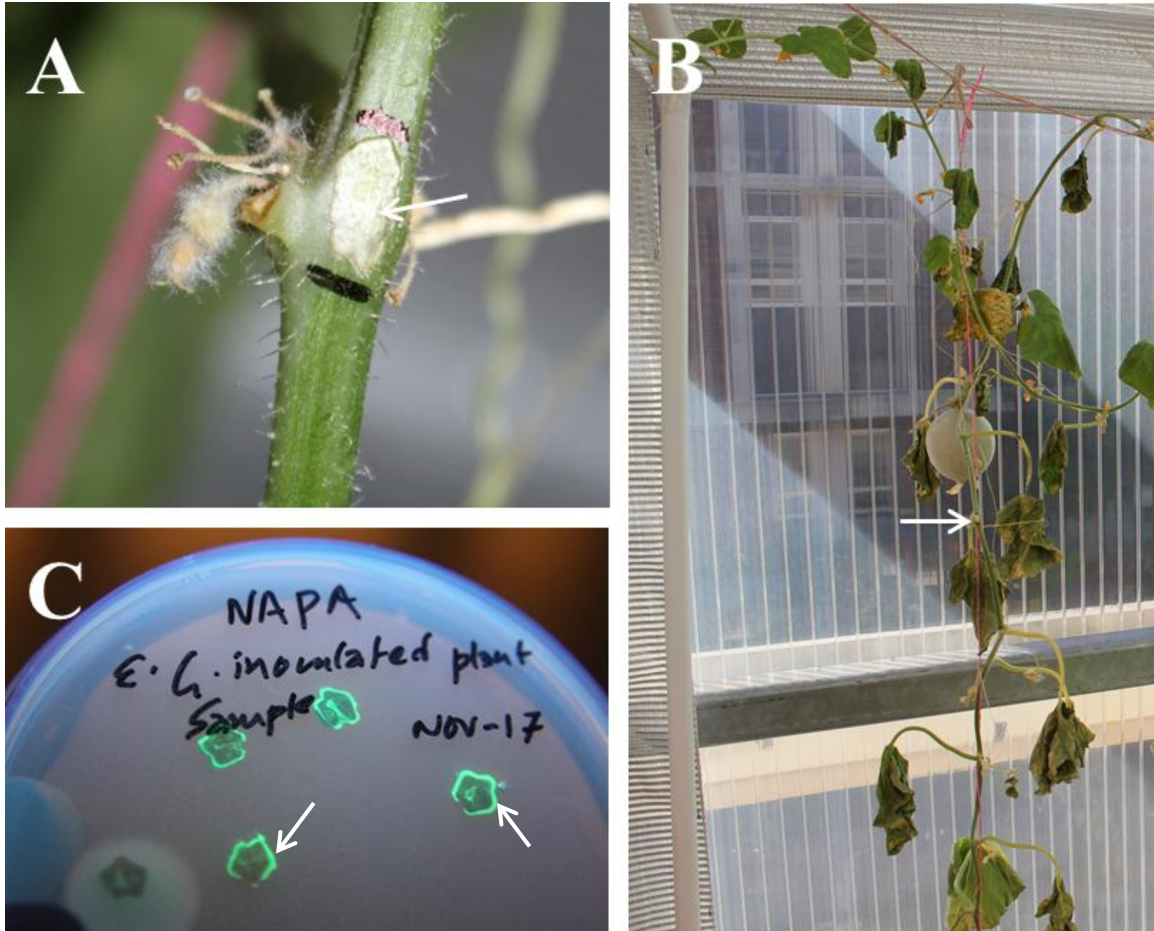

Figure 5. Inoculation of cantaloupe plant with GFPuv tagged *E. tracheiphila*. A. Site of *E. tracheiphila* inoculation, B. Wilting of plant after *E. tracheiphila* inoculation (arrow showing site of inoculation), and C. Recovery of *E. tracheiphila* from stem samples of wilted plant (green fluorescing bacteria on arrow heads), incubated on ampicillin-amended nutrient agar and observed under UV light.

Figure 6. Evidence of *Erwinia tracheiphila* entry into cantaloupe plants following flower interior introduction. A. Watersoaked lesions appear on developing fruits and peduncles collapse; B. Wilted vine; C. Bacteria stream from the freshly cut stem; D. Impaired netting on mature fruit; E. Internal tissue of symptomatic fruit showing presence of *E. tracheiphila* on nutrient agar plate; F. A normal fruit; G. PCR results showing 68 bp amplicon, from tissue with watersoaked lesion.

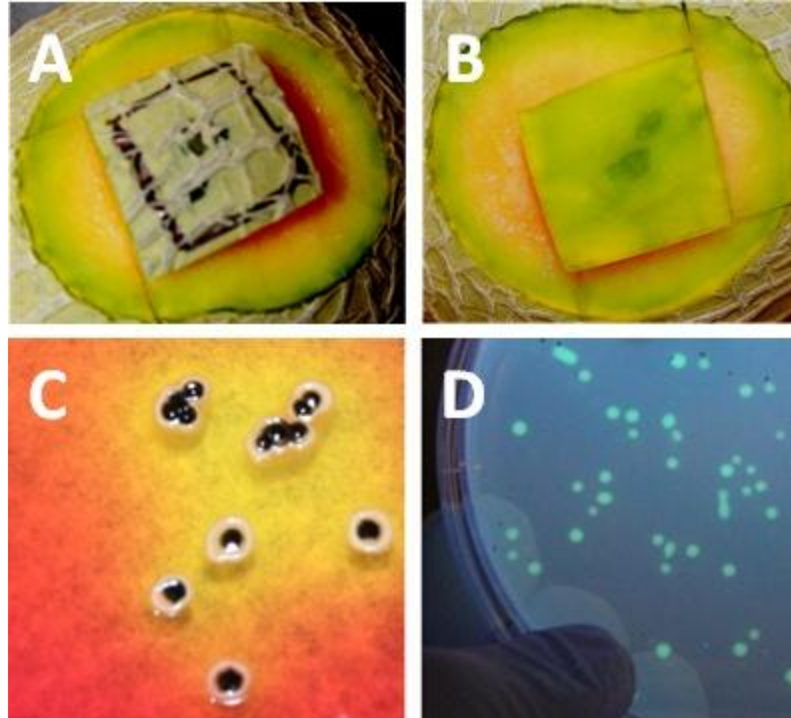

Figure 7. Cantaloupe fruit rind surface inoculated with a mixture of *S. enterica* and *E. tracheiphila* at the time of natural fruit cracking and sampled at fruit maturity (40 DPI). A. *E. tracheiphila* lesion, on the fruit rind, just before rind layer extraction, B. Sub-rind mesocarp with *E. tracheiphila* lesion, suggesting bacterial traversal through the outer rind, C. Black colonies of *S. enterica*, recovered from fruit rind, observed on XLD plate, and D. Recovery of *E. tracheiphila* from fruit with watersoaked lesion, observed under UV light on ampicillin amended nutrient agar.

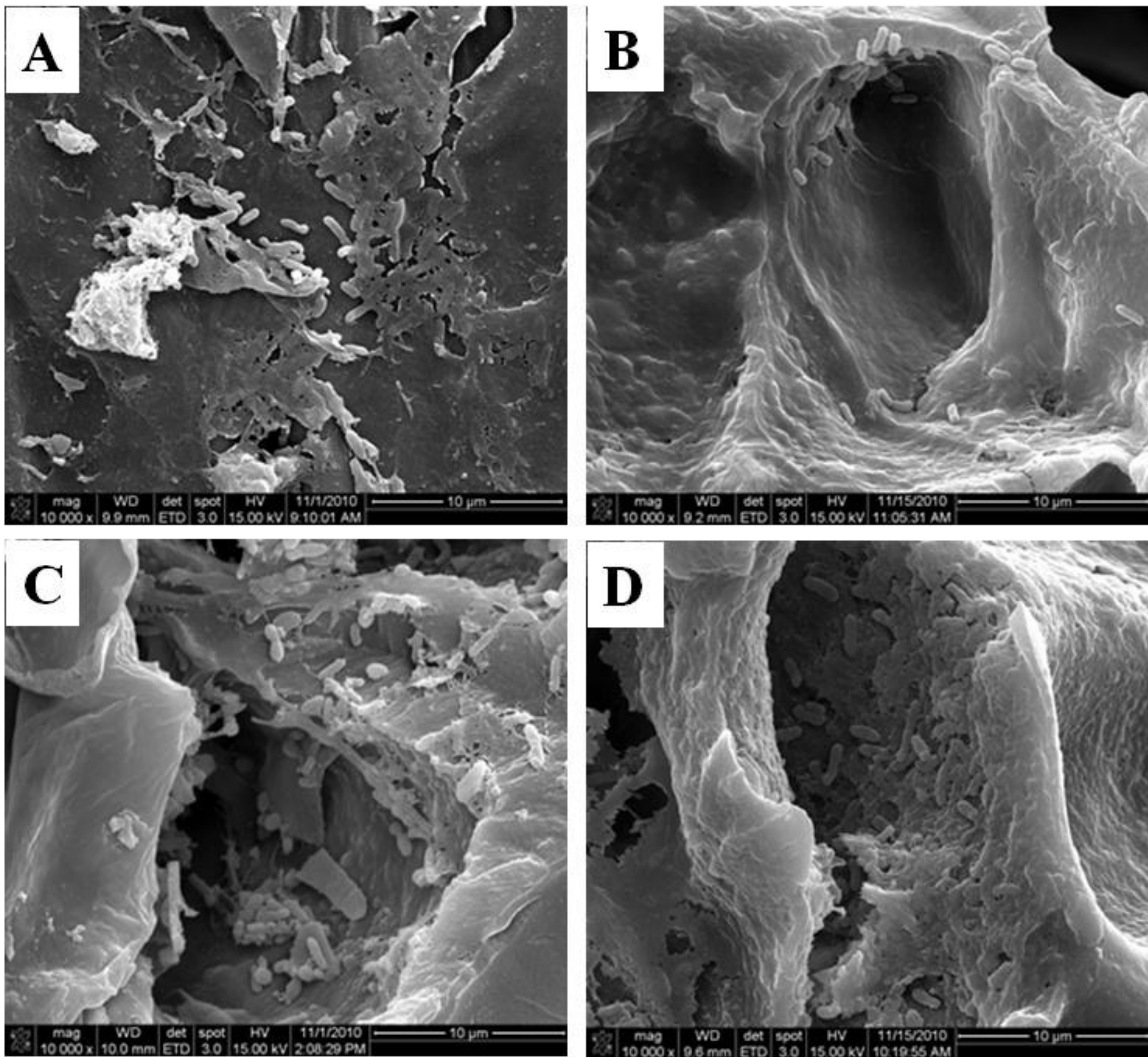

Figure 8. Interaction of *Salmonella enterica* Poona, alone or in the presence of *E. tracheiphila,* on the natural fruit cracks, sampled over time. Fruit crack inoculated with *S. enterica* only and sampled 7 days post inoculation (DPI) (A) and 19 DPI (B). Fruit crack inoculated with a mixture of *S. enterica* + *E. tracheiphila* and sampled at 7 DPI (C) and 19 DPI (D).

Table 1. Mean percentage of fruits positive for *Salmonella* on fruit rind and sub-rind mesocarp, immediately below the rind layer, of fruit rind initially inoculated with *Salmonella* only or *Salmonella* + *E. tracheiphila*, sampled at 7 and 19 days post inoculation (DPI).

Sampling Time	Fruit inoculation with *S*.P. only			Fruit inoculation with *S*.P. + *Et*		
	Total Fruits	% *S*.P. recovery		Total Fruits	% *S*.P. recovery	
		Rind	Sub-rind mesocarp		Rind	Sub-rind mesocarp
7 DPI	5	100	0	5	100	20
19 DPI	5	80	0	7	86	14

VITA

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Thesis: FATE OF *SALMONELLA* INTRODUCED TO CANTALOUPE THROUGH NATURAL FRUIT CRACKS AND FLOWERS, ALONE OR IN THE PRESENCE OF THE PLANT PATHOGEN *ERWINIA TRACHEIPHILA*

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