ABSTRACT

CARLSON, ALICAIN SUZANNE. Evaluation of Bacteria Species, Solution pH, and Differential Gene Expression on Cut Flower Postharvest Longevity. (Under the direction of Dr. John M. Dole.)

Cut flower postharvest longevity is affected by many factors. This research seeks to address vase solution micro-organisms, vase solution pH, and gene expression to improve vase life. Bacterial growth in vase solutions can lead to stem vasculature blockage causing water stress symptoms that reduce postharvest quality. However, managing the species of bacteria may be more important than maintaining low microbial loads. This research isolated and identified ten bacteria species associated with cut Zinnia postharvest, including Pseudomonas fulva, Serratia ficaria, Rhizobium radiobacter, Chryseobacterium sp., Pantoea ananatis, Bacillus pumilus, Chryseobacterium daejeonense, Brevundimonas sp., Escherichia coli K 12, and Pseudomonas marginalis, and investigated the effects of pure cultures on cut Zinnia ‘Benary’s Giant Wine’. Stems inoculated with P. fulva and E. coli K 12 had significantly greater vase lives of 9.5 and 9.4 d, respectively compared P. marginalis, P. ananatis, R. radiobacter, and a nutrient broth control (7.0, 6.9, 6.8 d, and 7.3, respectively). The other bacteria species resulted in an intermediate vase life, which were statistically similar to each other and to the deionized water control (8.6 d). There were no significant differences in water uptake and vase solution bacteria concentrations among all treatments.

Vase solution pH is another important postharvest factor and low pH may improve stem water uptake, reduce embolization, and slow bacterial growth. In these studies we investigated the effect of solution pH (acidic, neutral, basic, and a commercial preservative) and the addition of two different bacteria strains from previous studies, one detrimental (P. marginalis) and the other beneficial (E. coli K 12), on Zinnia postharvest, including the number of days to water stress (DTWS),
Non-surface sterilized stems held in solutions with only the beneficial bacteria or only preservative had the most DTWS (8.0 d). The number of bacteria inside and outside the stem were lowest in the preservative compared to the other solutions. Among the treatments with surface sterilized stems, acidic solutions had a significantly lower percent loss in conductivity (64%) when the lower 5 cm of the stem was removed, compared to the preservative (87%) and neutral (83%). These studies showed bacteria species in the vase solution have an effect on postharvest, and by combining low pH and beneficial bacteria an effective novel organic floral preservative may be possible.

Analysis of the changes in global gene expression patterns during the onset of peduncle bending (bent neck) and petal blueing in cut *Rosa hybrida* ‘Freedom’ identified 297 and 337 differentially expressed genes compared to healthy tissues. Differential expression was also performed comparing healthy neck tissues of two cultivars, ‘Freedom’ and ‘Forever Young’, the latter with a significantly longer vase life in previous studies, which identified 818 differentially expressed genes. The data suggest that bent neck may be due to cell wall degradation of the peduncle related to general senescence and petal blueing may be due to sugar deprivation. All tissues had significant expression of genes related to various stressors, including wounding, pathogens, and water deprivation. This work will provide further assistance to postharvest scientists creating novel vase solutions, breeders using marker assisted selections, and molecular geneticists transforming rose. In total, this dissertation provides valuable information about possible novel ways to extend postharvest life of significant cut flower species based on physiological and molecular data.
Evaluation of Bacteria Species, Solution pH, and Differential Gene Expression on Cut Flower Postharvest Longevity

by
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DEDICATION

This dissertation is dedicated to my grandmother,

Theresa Mary (Mahosky) Carlson

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BIOGRAPHY

Alicain Suzanne Carlson was born on January 17, 1986 to Gilbert and Barbara Jean Carlson in Wellsboro, Pennsylvania. Alicain spent her childhood travelling the country thanks to her parents’ careers with the United States Army. She completed her Bachelor of Science degree in Biological Sciences at Virginia Tech in 2008. As an undergraduate, Alicain was a research assistant in Dr. Donald Cherry’s Aquatic Ecotoxicology laboratory and a teaching assistant for Dr. Alan R. McDaniel’s Floral Design class. She also worked as a grower and floral designer at Lynnvale Studios, a cut flower farm in Gainesville, Virginia owned by Andrea Gagnon. Alicain was accepted into the graduate program at North Carolina State University (NCSU) in 2008 studying cut flower production and postharvest under the direction of Dr. John Dole and completed her Masters of Science in Horticultural Science in 2010. She continued her studies at NCSU to earn a Ph.D. in 2014.
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CH. 1: LITERATURE REVIEW

Introduction

The short vase life that results from poor handling cut flowers taints the perceptions of consumer to think that all flowers will only last a few days. These thoughts deter the future purchase of flowers for special occasions and daily enjoyment, and hold down flower purchases. The current wholesale value of domestically produced cut flowers was $342 million in 2012; 8.3% of the total $4.13 billion value of all floricultural crops (USDA, 2013). *Rosa hybrida* L. continues to be one of the most important ornamental crops in the world. In 2012, the total wholesale value of cut roses in the United States was $16.8 million (USDA, 2013). Additionally, the US imported $615.7 million and exported $25.8 million in cut flowers in 2012 with $367.3 million of that being from cut roses (Global Agricultural Trading System, USDA). Improved postharvest handling of cut flowers would most likely result in higher cut flower sales.

Advances in production, harvest, and postharvest techniques have considerably extended the vase life of many cut flower species. More information is available regarding the best cultivars for each grower’s climate and unique production situation. Many commercial postharvest solutions contain carbohydrates, acidifiers, biocides, anti-ethylene agents, and plant hormones that improve vase life of many cut flower species. The trend of buying local products, including locally produced cut flowers, has also had a positive impact on postharvest life by reducing shipping distances. Some flowers, like roses, are more economically produced in South America, but postharvest quality tends to suffer due to long, strenuous shipping conditions. Despite all of these advancements in extending vase life, some issues still remain. In these studies we chose to focus on two species, *Rosa hybrida* and *Zinnia elegans* L. Cut roses have postharvest issues that reduce vase life, including bent neck
(peduncle bending) and petal blueing (Zieslin, 1989). Both symptoms generally occur before the petals visibly wilt beyond levels that would be unacceptable to consumers. *Zinnia elegans*, an important field grown cut flower crop, produces flowers that have water balance related vase life problems, where transpiration exceeds uptake causing tissue wilt and desiccation (Twumasi et al., 2005) that may be influenced by bacteria. *Zinnia* is commonly referred to as a “dirty flower” in the industry, because it carries much bacteria with it from the field, quickly dirtying the water the stems are held in. The unique postharvest issues and industry importance of these two species make them ideal to study.

**Effects of bacteria concentrations and species on postharvest quality**

One of the main issues in cut flower postharvest is controlling microorganism growth. The importance of clean cutting instruments, buckets, and solutions are stressed in many postharvest resources (Armitage et al., 2004; Dole and Wilkins, 2005; Hunter, 2000; Sacalis and Seals, 1993). Research has shown that bacterial growth in vase solutions can lead to stem vasculature blockage causing petal and leaf wilt, bent neck, or similar water stress related symptoms that reduce vase life (Put, 1990; Put, 1986; van Doorn et al., 1991; Zagory and Reid, 1986; de Witte and van Doorn, 1988). The effects of bacteria concentrations in vase solutions differ with cut flower species. De Witte and van Doorn (1988) found that roses inoculated with \(10^7\) cfu·mL\(^{-1}\) of several different bacteria strains reduced water uptake by the first day. Put and Jansen (1989) found cut rose vase life was reduced by bacteria concentrations as low as \(10^5\) cfu·mL\(^{-1}\), while Jones and Hill (1993) found *Dianthus caryophyllus* L. ‘Medea’, *Iris* L., *Alstroemeria* L., and *Tulipa* L. tolerated bacterial counts up to \(10^8\) cfu·mL\(^{-1}\). Therefore, the number of bacteria in the vase solution may not be the primary cause of wilting or shortened vase life in all cut flower species.
Controlling the species of bacteria present in the vase solution may also be important. While many bacteria in vase solutions reduce vase life, research has identified bacteria that have little effect (de Witte and van Doorn, 1988; Jacob and Kim, 2010; Putt, 1986; van Doorn et al., 1991; Zagory and Reid, 1986). Some bacterial species produce exopolysaccharides that may clog the vasculature (Put, 1990), enzymes that degrade plant tissue (Membre and Burlot, 1994), and hormones, like ethylene, that cause senescence (van Doorn, et al., 1991). Several genera of bacteria have been identified from cut flowers, including: Alcaligenes, Pseudomonas (de Witte and van Doorn, 1988; Put, 1990), Acetinobacter, Achromobacter, Bacillus, Chromobacterium, Citrobacter, Enterobacter, and Erwinia (Put, 1990). De Witte and van Doorn (1988) identified bacterial strains from ‘Sonia’ roses as Alcaligenes faecalis or belonging to the genus Pseudomonas. None of them showed any pectinolytic activity that would allow them to digest cell walls. De Witte and van Doorn (1988) noticed extensive extracellular polysaccharide production in some strains and less in others. The role of extracellular polysaccharides in vascular blockage is not known, but is likely to be an important factor in vessel plugging. The bacteria strains currently documented have been isolated from only a few cut flower species: Chrysanthemum, Gerbera (Put, 1990), D. caryophyllus (Zagory and Reid, 1986), and Rosa (de Witte and van Doorn, 1988; Put, 1990; van Doorn et al., 1991; van Doorn et al., 1989); all commercially significant imported cut flower species. No literature could be found on bacteria isolated from Zinnia.

**Effects of vase solution pH on postharvest life and bacteria growth**

Solution pH has been found to be an important factor in vase life for many species of cut flowers, with low pH (3.0-4.0) increasing the vase life of Dendranthema L. (Carlson and Dole, 2013), Helianthus L. (Stevens et al., 1993; Carlson and Dole, 2013), and Rosa L. (Regan and Dole, 2010;
Conrado et al., 1980; Durkin, 1979). Carlson and Dole (2013) found that vase life of cut *Zinnia* was not affected by vase solution pH. Vase water can be acidified with citric acid, aluminum sulfate, or other strong acids such as hydrochloric acid (HCl). Citric acid is a safe organic acid (Gast, 2000) and is much cheaper and more effective than aluminum sulfate, which also induces stomatal closure reducing transpiration and improving water balance (Sacalis and Seals, 1993). Commercial preservative products, including hydrating, holding and vase solutions, also contain acidifiers. The increase in vase life may be from an improvement of stem water uptake (Gast, 2000; Sacalis and Seals, 1993), slowing of bacterial growth in the vase solution (Conrado et al., 1980; van Doorn and Perik, 1990), and/or reduction of embolization (Durkin, 1979).

**Improved water uptake and Hydraulic Conductivity**

Inadequate water uptake is one of the main reasons for inferior cut flower vase life and may be caused by obstruction of xylem vessels due to microbial growth, formation of tyloses, deposition of materials in the lumen of xylem vessels, and the presence of air emboli in the vascular system (van Doorn, 1997). Carlson and Dole (2013) found solution uptake to be slightly higher in acidic solutions for *Dendranthema* and *Dianthus*, but this difference was not statistically significant from basic and neutral solutions and would not fully account for the difference in vase life. Sperry et al. (1988) observed that solutions with a pH below 3.0 prevented long-term declines in hydraulic conductivity, apparently by limiting bacterial growth. As the concentration of bacteria in the solution increases above 10^6, the hydraulic resistance in the stem increases sharply (van Doorn and de Witte, 1991). Van Doorn et al. (1989) found that the basal 5 cm of the stems limited the flow of water and that this blockage was correlated with a high number of bacteria in this stem segment. Van Doorn and Perik (1990) found that while the vase water of cut flowers may be sterile, a considerable
number of bacteria are present in the xylem vessels and the cut surface and hydraulic conductance of the stem decreased when the number of bacteria was $10^6$ cfu·g$^{-1}$ fresh weight or higher. Not all bacteria are killed by low pH (Hosein et al., 2011), which would allow for subsequent proliferation if the pH was not kept constantly low.

Low pH solutions are proposed to be beneficial for cut stems by helping to prevent and repair xylem air occlusions. There is considerable variation in vulnerability to cavitation among species and among different parts of the plant. Vulnerability to cavitation determines the capacity for water transport (Tyree and Sperry, 1989). The mechanisms by which embolisms are formed and repaired are not fully understood. Vulnerability curves have not been determined for Zinnia elegans cut stems. Xylem cavitation in cut stems of Rosa ‘Samantha’ was initiated at low stem water potentials of -0.2 to -0.4 MPa (Dixon et al., 1988). Stem hydraulic conductivity can be measured to determine what percentage of the stem is embolized. Durkin (1979) found that acidifying filtered water results in the same hydration effects as cutting stems under water, suggesting that acidified water may improve water uptake by reducing emboli; the mechanism for how this works is unknown. Van Ieperen et al. (2002) investigated air aspiration into cut stems and its effects on hydraulic conductance and found that during the first few seconds after placing a cut stem in water, initially air-filled vessels at the cut surface partly refill with water. Reconnections are then established between the vase water and the non-cut water-filled xylem vessels just above the cut surface and hydraulic conductance is partly recovered. During the following hours, air partly or completely dissolves into the surrounding water in the stem and hydraulic conductance recover gradually takes place. They also found that hydraulic conductivity repair occurs more readily in stems with smaller diameter vessels.
Marousky (1971) found that stems held in a pH of 3.0 had a higher hydraulic conductance than stems held in a pH of 6.0; no bacteria were found in the vase solution, so a bacterial effect was excluded. Durkin (1980) hypothesized that the acidified water enhanced lateral water movement through vessel walls, possibly by breaking calcium pectate bonds. Citric acid was found to extend the vase life of *Acacia* stems, increase hydraulic conductance, improve water potentials, and reduce the number of audible acoustic emissions (AAE) (Williamson and Milburn, 1995). Citric acid may degrade pit membranes, which would account for the lower numbers of detectable AAE, because the potentially cavitaatable compartments would fuse together (Williamson and Milburn, 1995). Zimmerman (1978) showed that drawing distilled water through stem segments progressively decreased conductance and this reduction can be eliminated by using tap water or a dilute osmoticum of 10 mM sodium chloride (NaCl).

Plants also have reactions to wounding that primarily serve to impede the entry of microorganisms into the opened tissue. Such responses could subsequently cause a decline in water uptake. Stems of *Dendranthema ‘Viking’* were found to form xylem blockages, which were mediated by 5 hour pretreatments in low pH solutions, involving oxidative reactions in response to wounding (van Doorn and Cruz, 2000).

*Probiotics and organic preservatives*

Biocontrol bacteria usually confer their positive effects by preventing growth and proliferation of phytopathogens by producing toxic metabolites, competing for nutrition and colonization sites, and triggering defense response-related genes in plants (Gao et al., 2012). *Pseudomonas fulva* is known to be antagonistic to fungal and bacterial pathogens of rice (Xie et al., 2003) and involved in plant pathogenic fungi elimination during the filtration process used in soilless
tomato culture (Renault et al., 2007). The effects that bacteria have on plants could also be due to their products rather than the live cultures. There are some gram-negative plant pathogenic bacteria species that produce Harpin protein that is theorized to trigger systemic acquired resistance (SAR) immune response in plants when applied during plant production (Zhang et al., 2007). This protective response makes the plant resistant to a wide range of fungal, bacterial, and viral diseases with the response continuing postharvest. Zhang et al. (2011) found that the overexpression of Harpin-encoding genes in rice improved its tolerance to abiotic stresses, such as drought. It has not been tested whether Harpin proteins added to cut flower vase solution improves vase life.

As the number of organic cut flower farms increases, the need for an organic floral preservative product that effectively controls microbial growth also increases. Commercial floral preservatives have three general components: an acidifier, a biocide, and a carbohydrate (sugar). Certified organic sources of the acidifier and carbohydrate are relatively easy to obtain, but a biocide is difficult. Beneficial bacteria could be added as an organic probiotic to vase solutions to improve vase life.

Postharvest problems of cut Rosa

Cut roses have a number of postharvest issues that reduce their vase life, which is very important factor in consumer perception of value. The most common postharvest problems for cut roses are bent neck and petal blueing (Zieslin, 1989). Both symptoms generally occur before the petals visibly wilt beyond levels that would be unacceptable to consumers. Bent neck may be related to water relations problems from a lack of water uptake caused by obstruction of xylem vessels due to microbial growth, formation of tyloses, deposition of materials in the lumen of xylem vessels, and the presence of air emboli (van Doorn, 1997). Petal blueing is hypothesized to be
caused by an increase in the vacuolar pH causing the anthocyanins to change colors (Marousky and Carlyle, 1985; Asen et al., 1971). Commercial postharvest solutions are available that attempt to ameliorate the problems by including a biocide, lowering the pH, and providing stems with carbohydrates and hormones. De Witte and van Doorn (1988) found floral preservatives (Chrysal and Aadural) to prevent development of high bacterial numbers in vase water of rose and hypothesized that their effectiveness against bent neck may be partially due to the presence of an effective biocide. Stoltz (1956) showed petals did not blue or become deficient in carbohydrates when preservatives containing glucose or sucrose were used and hypothesized that protein hydrolysis occurred only under conditions of carbohydrate starvation. Borochov et al. (2006) found that when added to the vase solution, sucrose retarded and ABA promoted processes associated with senescence, including petal wilt, increased pH in petal tissue, petal blueing, and a decrease in protein content of petals. They suggested that ABA accelerates senescence of cut roses by promoting petal growth and respiration, thus decreasing the carbohydrate level in the petals and triggering the chain of metabolic processes leading to aging. While these studies provided much information about petal blueing and bent neck, a more effective approach may come from understanding the differential gene expression changes during the onset of these symptoms using next-generation sequencing (NGS) technologies such as RNA-seq on highly efficient Illumina sequencing platforms.

Next generation sequencing technologies

The development and emergence of NGS technologies in the last few years have had significant impacts on all biological disciplines (Egan et al., 2012). Next generation sequencing methods have increased capabilities far beyond that of traditional Sanger sequencing (Sanger et al.,
1977), allowing millions of bases to be sequenced in one round at a fraction of the cost (Egan et al., 2012). An NGS method, RNA-seq has become the technology of choice for whole transcriptome sequencing (Deng et al., 2011) allowing molecular geneticists to explore transcriptomes of basically any species through de novo assembly without the need for a reference genome (Ekblom and Galindo, 2011; Jain, 2011), making it particularly useful in roses as the rose genome has not been completely sequenced. Currently, there are sequences of only 5,000 rose genes in the public databases (Dubois et al., 2012). Most commercially bred rose varieties are tetraploid, which makes the traditional breeding process slower, especially for complex and combined traits (Bendahmane et al., 2013). The haploid genome size (as determined using flow cytometry) of rose is estimated to be between 300 and 585 million basepairs (Bendahmane et al., 2013). RNA-seq is more accurate and captures a broader range of expression levels compared to microarrays, because it does not rely on hybridization (Marioni et al, 2008; Fu et al., 2009). The absolute measurement of gene expression using RNA-seq provides greater quantitative and qualitative insight, and accuracy than microarray especially in detecting low-expressed genes, alternative splice variants, and novel transcripts (Jain, 2011). Illumina RNA-seq technologies have improved over the last few years, as well as the algorithms and chemistry in the newer HiSeq 2500 sequencers that now can produce up to 185 million single reads per lane as opposed to 45 million for the Illumina GAIIx sequencer. Illumina technologies have very fine resolution, down to the base pair, and homopolymer errors are not problematic like they can be for Roche 454 pyrosequencing. With the improved sequencing technologies come challenges in managing and analyzing the vast amounts of data. Several programs have been developed that are effective in analyzing differential expression using the Tuxedo Pipeline (Trapnell et al., 2012). As the costs and capabilities of these technologies continue
to improve, the possibilities for the applications of these technologies becomes almost limitless (Egan et al., 2012).

Gene expression changes causing bent neck and petal blueing

Limited information is known about the molecular mechanisms that control bent neck and petal blueing in rose. Zhu (2010) studied global gene expression during bent neck for *Rosa ‘Freedom’* and concluded that bent neck may be caused by water stress and cell wall degradation of the peduncle. For example, increased expression of β-galactosidase and β-xylosidase was found in bent neck compared to healthy tissue, which may reduce peduncle rigidity due to degradation of cell wall components (Trainotti et al., 2001; Martinez et al., 2004). Zhu (2010) also found high expression of laccase, which can be induced by ethylene (Ahmadi et al., 2009) in ‘Freedom’ bent neck samples. Technologies have significantly improved since 2010, so further study of this topic should provide more useful information. Such information could be used by breeders using marker-assisted selections to prevent these symptoms. Molecular markers allow breeders to rapidly screen a large number of lines for markers associated with traits of interest, allowing the subsequent selection of relevant molecular markers and thus specific introgression of single genomic loci (Dubois et al., 2012). Information could also be used to create novel postharvest treatments or genetically modified plants to prevent bent neck and petal blueing.

Vase solution pH, interactions with micro-organisms, and genetic factors play important roles in extending vase life. This research seeks to enhance postharvest quality of cut flowers by developing an understanding of the roles of bacteria in vase solutions, improving cut stem water relations using pH and probiotics, and exploring the genetic components controlling postharvest quality.
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**Abstract**

Bacterial growth in vase solutions can lead to stem vasculature blockage causing petal and leaf wilt, bent neck, or similar water stress related symptoms that reduce vase life (Put, 1990; Put, 1986; van Doorn et al., 1991; Zagory and Reid, 1986; de Witte and van Doorn, 1988). However, controlling the species of bacteria present in the vase solution may be more important than just the numbers. We have isolated and identified ten bacterial species associated with cut \textit{Zinnia} postharvest, including: \textit{Pseudomonas fulva}, \textit{Serratia ficaria}, \textit{Rhizobium radiobacter}, \textit{Chryseobacterium sp.}, \textit{Pantoea ananatis}, \textit{Bacillus pumilus}, \textit{Chryseobacterium daejeonense}, \textit{Brevundimonas sp.}, \textit{Escherichia coli} K 12, and \textit{Pseudomonas marginalis}, and investigated their effects on vase life of cut \textit{Zinnia} ‘Benary’s Giant Wine’. Cut flowers inoculated with \textit{P. fulva} and \textit{E. coli} K 12 had significantly greater vase lives of 9.5 and 9.4 d, respectively compared to \textit{P. marginalis}, \textit{P. ananatis}, \textit{R. radiobacter}, and the nutrient broth control (7.0, 6.9, 6.8, and 7.3 d, respectively). The other bacterial treatments resulted in slight differences in vase life, but generally had no statistically significant effect on vase life when compared to the deionized (DI) water control (8.6 d). There was
no significant difference in water uptake, percent incidence of termination criteria, change in vase solution pH and electrical conductivity (EC), or bacteria concentrations in the vase at termination among all treatments. This research shows that it is not necessarily the number of bacteria in the vase solution, but the bacterial species that has an effect on vase life. Knowing the effects of different bacteria can lead to novel postharvest treatments that can target certain detrimental bacteria and avoid or even inoculate with others that may have probiotic qualities.

1. Introduction

One of the main issues in cut flower postharvest is controlling microorganism growth. The importance of clean cutting instruments, buckets, and solutions are stressed in many general postharvest resources (Sacalis and Seals, 1993; Dole and Wilkins, 2005; Hunter, 2000; Armitage et al., 2004). Research has shown that bacterial growth in vase solutions can lead to stem vasculature blockage causing petal and leaf wilt, bent neck, or similar symptoms related to water stress that reduce vase life (Put, 1990; Put, 1986; van Doorn et al., 1991; Zagory and Reid, 1986; de Witte and van Doorn, 1988). The effects of bacteria concentrations in vase solutions differ with cut flower species. Put and Jansen (1989) found cut Rosa ‘Sonia’ vase life was reduced by bacteria concentrations as low as $10^5$ colony forming units (cfu)-ml$^{-1}$, while Jones and Hill (1993) found Dianthus caryophyllus L. ‘Medea’, Iris L., Alstroemeria L., and Tulipa L. to be tolerant to bacterial counts up to $10^8$ cfu-ml$^{-1}$. Therefore, the number of bacteria in the vase solution may not be the primary cause of wilting or shortened vase life in all cut flower species.

Controlling the species of bacteria present in the vase solution may be more important than just the concentration. Research on microbial growth in vase solutions has identified bacteria that decrease vase life or have little effect (Jacob and Kim, 2010; van Doorn et al., 1991; Zagory and Reid,
186, de Witte and van Doorn, 1988; Putt, 1986). Some bacteria species produce exopolysaccharides that can clog the vasculature (Put, 1990), enzymes that degrade plant tissue (Membre and Burlot, 1994), and hormones, like ethylene, that cause senescence (van Doorn, et al., 1991). Several genera of bacteria have been identified from cut flowers, including: Alcaligenes, Pseudomonas (de Witte and van Doorn, 1988; Put, 1990), Acetinobacter, Achromobacter, Bacillus, Chromobacterium, Citrobacter, Enterobacter, and Erwinia (Put, 1990). However, the bacterial strains currently documented have been isolated from only a few cut flower species: Chrysanthemum, Gerbera (Put, 1990), D. caryophyllus (Zagory and Reid, 1986), and Rosa (de Witte and van Doorn, 1988; Put, 1990; van Doorn et al., 1991; van Doorn et al., 1989); all commercially significant imported cut flower species.

Zinnia elegans, an important field grown cut flower crop, produces flowers that have vase life problems related to water stress, where transpiration exceeds uptake causing tissue wilt and desiccation (Twumasi et al., 2005) that may be influenced by bacteria. Zinnia is commonly referred to as a “dirty flower” in the industry because it carries a lot of bacteria from the field quickly dirtying the water they are held in. This characteristic makes it an excellent species for bacterial studies. The objective of this research is to isolate and identify bacteria species associated with cut Zinnia postharvest and investigate the effects of pure cultures on vase life.

2. Materials and methods

2.1 Bacteria isolation and identification

Zinnia ‘Benary’s Giant Wine’ (Harris Seeds, Rochester, NY, USA) were planted on 21 May 2012 in Raleigh, NC in 1.2 x 30.5 m, loamy clay soil beds. On 10 July 2012, ten were stems harvested and held in sterilized DI water (pH 5.2, EC 0.00 dS·m\(^{-1}\)) and allowed to senesce in a postharvest
environment at 21 ± 2 °C at 40-60% relative humidity under approximately 20 µmol·m⁻²·s⁻¹ light for 12 h·d⁻¹ in an uncovered vase solution. After complete flower senescence (100% petal wilt and bent neck), the vase solution was stirred and ten 0.01 mL samples of vase water were plated on nutrient agar (Remel, Thermo Fisher Scientific, Lenexa, KS, USA) and incubated at 21 ± 2 °C for 48 h. The colonies were visually inspected and twelve single colonies were selected by differences in colony characteristics such as, color, shape, size, and texture and plated on nutrient agar. Pure colonies were obtained and identified using the BIOLOG Microlog3 (Version 5.2.2.33, Biolog, Hayward, CA, USA). If the bacterium could not be identified using the BIOLOG, 16S rDNA-based PCR sequencing was used. Eight distinct species were identified and used for these studies, including: *Pseudomonas fulva* (ZNB), *Serratia ficaria* (ZO), *Rhizobium radiobacter* (ZCE), *Chryseobacterium sp.* (ZY3), *Pantoea ananatis* (ZY2), *Bacillus pumilus* (ZWS), *Chryseobacterium daejeonense* (ZO2), *Brevundimonas sp.* (ZBO). One strain previously isolated from *Zinnia* in the Matthysse lab, *Pseudomonas marginalis* (Z7pg), and *Escherichia coli K12* (Hfr Hayes strain, EC39) from the Matthysse lab collection were also used in these studies for a total of ten bacteria species.

2.2 Vase inoculations

Cut stems of *Zinnia ‘Benary’s Giant Wine’* were harvested from field beds planted on 21 May 2012 (replication 1) and 15 May 2013 (replications 2, 3, and 4), placed into tap water (6.1 pH, EC 0.21 dS·m⁻¹), and allowed to hydrate for one hour before being placed into treatments. Stems were sorted into twelve groups of nine stems each, according to flower head size, and stems cut to 35 cm, labeled, and placed into bacteria treatments. All glassware and water were sterilized via autoclaving before inoculation. The stems were held in 500 mL of DI water plus 0.5 mL of inoculum. The bacteria for inoculation were cultured for approximately 4 d at 21 ± 2 °C in 10 mL of nutrient broth (Remel,
Thermo Fisher Scientific, Lenexa, KS, USA) on a shaker (Gyrotory water bath shaker, Model G76, New Brunswick Scientific Co., Inc., Edison, NJ, USA) to a concentration of approximately $10^8$ cfu·mL$^{-1}$, which was diluted to a final concentration of $10^5$ cfu·mL$^{-1}$ in the vases. A DI water and a DI water plus 0.5 mL nutrient broth were also included as controls. The nutrient broth control was included to control the possible effects of the nutrient broth in the bacteria inoculation treatments.

The experiment was replicated four times and each replication consisted of three vases per treatment and three stems per vase. The experiments were conducted on 11 Oct 2012, 16 July 2013, 10 Sept 2013, and 17 Sept 2013. This resulted in 36 stems per treatment total. Vases were arranged in a completely randomized design and placed in the same postharvest environment as above until termination. Stems were observed daily and terminated when they showed bent neck or 50% wilted, desiccated, or blue petals; symptoms were recorded for each stem as present or not present. Days to termination (vase life) and initial and final vase solution pH and EC were recorded. Samples were taken from vase solutions when the last stem in each vase was terminated and bacteria were quantified using serial dilutions up to $10^{-6}$ cfu·mL$^{-1}$ and plated onto nutrient agar media (Remel, Thermo Fisher Scientific, Lenexa, KS, USA) and incubated for 48 h at 21 ± 2 °C. All plates used during these studies included 0.1% cyclohexamide to prevent fungal growth. Water uptake was measured by weighing every vase at the beginning of the experiment and when the first stem of the entire study was terminated.

2.3 Statistical analysis

Data were analyzed using SAS/STAT software (Version 9.3, SAS Institute, Inc., Cary, NC) by Analysis of Variance (ANOVA) procedures using the General Linear Models (GLM) procedure and
means were separated using Tukey’s Studentized Range procedure at $\alpha = 0.05$. Vase was used as the experimental unit and blocked by replication.

3. Results and discussion

3.1 Bacteria isolation and identification

Most bacteria isolated from the vase water of cut Zinnia are species commonly associated with soil or aquatic environments. All but two (Brevundimonas sp., Chryseobacterium sp.) could be identified to species. Pseudomonas (de Witte and van Doorn, 1988; Put, 1990; Put and Jansen, 1989) and Bacillus (Put, 1990; Put and Jansen, 1989) have been previously identified and are commonly associated with cut flower vase solutions from Chrysanthemum, Gerbera (Put, 1990), and Rosa (de Witte and van Doorn, 1988; Put, 1990; Put and Jansen, 1989). However, several have not been previously isolated from vase solutions, including: Brevundimonas sp., Chryseobacterium sp., Chryseobacterium daejeonense, Pantoea ananatis, Rhizobium radiobacter, and Serratia ficaria.

3.2 Vase inoculations

There was no significant difference in water uptake due to the bacteria treatments (data not presented). Bacterial growth may not have been rapid enough in the time frame of a few days until the first stem in the entire study was terminated to allow significant differences in water uptake to be observed. Van Doorn et al. (1995) found that water uptake in cut D. caryophyllus was not inhibited until vase solution bacteria concentrations reached $10^8$ cfu·mL$^{-1}$ or higher. Bacteria concentrations in the vase solution at termination in our experiments ranged from $1.8$ to $9.0 \times 10^6$ cfu·mL$^{-1}$ and were not significantly different. Percent incidence of all the termination criteria (petal wilt, desiccation, blueing and bent neck) was not significantly different between the treatments (data not presented). Petal wilt was observed in nearly every stem because it is a common symptom
of cut flower senescence in *Zinnia*. Final vase solution pH and EC ranged from 6.2 to 6.4 and 0.03 to 0.05 dS·m⁻¹, respectively, with no significant treatment effect. Final vase solution EC increased very little from initial values, which indicates that there was little tissue breakdown and subsequent release of cell contents into the vase solution.

The cut *Zinnia* flowers inoculated with either *P. fulva* or *E. coli* K 12 had significantly greater vase lives of 9.5 and 9.4 d, respectively, compared to the nutrient broth control, *P. marginalis*, *P. ananatis*, *R. radiobacter*, and the nutrient broth control (7.0, 6.9, 6.8, and 7.3 d, respectively) (Fig. 1). The DI water control had a vase life of 8.6 d. The other five bacterial treatments had slight differences in vase life, but generally had no statistically significant effect on vase life when compared to the DI water control.

The nutrient broth control may have reduced vase life significantly because nutrient broth served as a source of nutrition for bacteria on the stem allowing rapid multiplication which could have negative consequences for the stem. Since no bacteria were inoculated with the nutrient broth control, an endogenous detrimental bacterium may have proliferated. The number of bacteria in the nutrient broth control was similar to all the other treatments indicating that rapid proliferation had to occur for it to match levels in all the other treatments that were inoculated at 10⁵ cfu·mL⁻¹.

Investigating the characteristics of the individual bacteria species may give some indication of the effects they are having on the stems postharvest. Two of the three bacteria that had the shortest vase lives are known phytopathogens, which may account for the slight reduction in vase life. *P. marginalis* produces pectinolytic enzymes that breakdown the middle lamella and cells walls of plants tissues (Membre and Burlot, 1994) and *P. ananatis*, which is known to cause center rot in
onion seed (Walcott et al., 2002) and disease in rice plants (Cother et al., 2010). The bacterium that resulted in the shortest vase life, *R. radiobacter* (formerly *Agrobacterium tumefaciens*, commonly used in plant transformation) is not considered phytopathogenic, but had significant negative effects on vase life.

The five bacteria that resulted in vase life very similar to the control are generally not phytopathogenic and tended to have known benefits to other plant species or no effects. *S. ficaria* is usually associated with fig culture and is used as a biocontrol agent for *Phytophthora* in angelica tree (*Aralia elata*) (Okamoto et al., 2000). *B. pumilus* has been found to have anti-fungal properties (Bottone and Peluso, 2003). *Chryseobacterium daejeonense* has been previously isolated from greenhouse soils in Korea and was found to have no pectinolytic and cellulyotic characteristics (Kim et al., 2006). *Chryseobacterium sp.* are known to have cellulytic (Luis et al. 2004), lipolytic, and strong proteolytic activity and degrade carbohydrates (Vandamme et al., 1994). *Brevundimonas sp.* have been found in aquatic and soil habitats depending on the species and were found by Park et al. (2008) to promote the growth of cultured *Chlorella ellipsoidea* algea. Without knowing the particular species of both these isolates it is difficult to speculate on the specific effects they could be having on the stems.

The two species that had a slight increase in vase life over the DI water control have positive qualities that may account for their effects. Biocontrol bacteria usually confer their positive effects by preventing growth and proliferation of phytopathogens by producing toxic metabolites, out-competing for nutrition and colonization sites, and triggering defense response-related genes in plants (Gao et al., 2012). *P. fulva* is known to be antagonistic to fungal and bacterial pathogens of rice (Xie et al., 2003) and produces siderophores which sequester iron from other potentially
pathogenic bacteria and fungi (Renault et al., 2007). *E. coli* K 12 is a debilitated non-pathogenic laboratory strain of *E. coli* that has a safe history of commercial use and no known adverse effects on plants (US Environmental Protection Agency, 1997). Jeter and Matthysse (2005) found that *E. coli* K 12 had little to no capacity to bind to plant surfaces when introduced to alfalfa sprouts. *E. coli* K 12 may be out-competing other bacteria by reducing nutrients available in the solution to other possibly detrimental bacteria. However, the number of *E. coli* colonies in final vase solution bacteria counts were not recorded. Final bacteria concentrations and vase solution pH and EC’s were not significantly different from other bacteria treatments so the mechanism used by *E. coli* K 12 to increase vase life remains unknown.

The effects that bacteria have on plants could also be due to their products rather than the live cultures. There are some gram-negative plant pathogenic bacteria species that produce Harpin protein that is theorized to trigger systemic acquired resistance (SAR) immune response in plants when applied during plant production (Zhang et al., 2007). This protective response makes the plant resistant to a wide range of fungal, bacterial, and viral diseases with the response continuing postharvest. Zhang et al. (2011) found that the overexpression of Harpin-encoding genes in rice improves its tolerance to abiotic stresses, such as drought. It has not been tested whether Harpin proteins added to cut flower vase solution improves vase life or whether any of the bacteria tested in these studies produce Harpin protein.

Our results are similar to those from other studies that found most bacteria to have little effect and a few to have negative effects on vase life. Van Doorn et al. (1991) isolated twenty strains from *D. caryophyllus* that had no effect on senescence, at initial concentrations of about $10^5$ cfu·mL$^{-1}$, but found only two strains that reduced vase life. One of the two strains produced measurable
amounts of ethylene. Mayak et al. (1977) also found a two day decrease in vase life when about $10^5 \text{ cfu·mL}^{-1}$ of a mixed bacterial population (species unknown) were included in the vase water of ‘White Sim’ carnations. This is in contrast to studies by van Doorn et al. (1991) that used the same concentrations, but shows the importance of the bacteria species composition. When a mixed bacterial population is included in the water, one or more of the potentially harmful strains may reach a concentration high enough to have a negative effect (van Doorn et al., 1991). This may be similar to the effect of the nutrient broth control in our work, which was essentially inoculated by the stem with a mix of random bacteria.

Not only do the different bacterial species have differing effects on vase life, but the effects may also be cut flower species dependent. Zagory and Reid (1986) found that a bacterial strain (described as a fluorescent pseudomonad) isolated from *D. caryophyllus* vase solution did not reduce vase life of *D. caryophyllus* or *Chrysanthemum*, but did effect *Rosa*, all at concentrations of $10^6 \text{ cfu·mL}^{-1}$.

**4. Conclusion**

The pure cultures isolated from *Zinnia* had differing effects on postharvest life of cut *Zinnia* stems. Some had negative effects, others had no effect, and a couple seem to increase it. This research shows that it is not necessarily the number of bacteria in the vase solution, but the bacteria species present in the vase solution that has an effect on vase life. Knowing the effects of different bacteria can lead to novel postharvest treatments that can target certain detrimental bacteria and inoculate with or avoid others that may be beneficial. These bacteria species should be tested on other cut flower species to see if they have similar effects. *E. coli* K 12 and *P. fulva* may have the potential to be used as an additive to vase solutions to improve cut *Zinnia* vase life, possibly as an
organic alternative to commercial floral preservatives, and should be trialed on more species of cut flowers and in different concentrations. The products from the bacteria and/or inert bacteria should also be tested to see if they have similar effects.

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References


Put, H.M.C. 1990. Micro-organisms from freshly harvested cut flower stems and developing during the vase life of chrysanthemum, gerbera and rose cultivars. Scientia Hort. 43:129-144.


Figure 1. Vase lives of cut Zinnia stems in vase inoculation treatments of various bacteria species, *Pseudomonas fulva* (ZNB), *Serratia ficaria* (ZO), *Rhizobium radiobacter* (ZCE), *Chryseobacterium sp.* (ZY3), *Pantoea ananatis* (ZY2), *Bacillus pumilus* (ZWS), *C. daejeonense* (ZO2), *Brevundimonas sp.* (ZBO), *Escherichia coli* K12 (EC39), and *Pseudomonas marginalis* (Z7pg) and control solutions, deionized (DI) water (DIC) and DI water plus 0.5 mL nutrient broth (NBC). Columns with the same letter are not significantly different according to Tukey’s Studentized Range procedure at $\alpha = 0.05$.

Calculated from the means of 36 stems per treatment from four replications ($P<0.0001$).
Abstract

Vase solution pH is an important factor in postharvest life for many species of cut flowers and low pH may improve stem water uptake, reduce embolization, and slow bacterial growth. Bacterial growth in vase solutions can lead to vasculature blockage and cause petal and leaf wilt, bent neck, tissue desiccation, or similar water stress related symptoms. In these studies we investigate solution pH and the addition of two different strains of bacteria, one known to decrease vase life and another that was hypothesized to increase vase life, to vase water of *Zinnia elegans* L. ‘Benary’s Giant Wine’ and observe their effects on number of days to water stress (DTWS), stem hydraulic conductivity, and bacteria counts inside and outside the stem. The detrimental bacterium used in these studies was *Pseudomonas marginalis* and the proposed beneficial bacterium was *Escherichia coli* K 12. The non-sterile stems/control solution/beneficial bacteria and non-sterile stems/preservative solution/no bacteria treatments had the most DTWS of 8.0 d. The sterile stems/control solution/beneficial bacteria and sterile stems/basic solution/no bacteria treatments
had the least DTWS of 5.5 d and 5.8 d, respectively. The numbers of bacteria inside and outside the stems were significantly lower in the preservative solution compared to acidic, basic, and control solutions. The vase solution pH of the acidic solution increased the most by 3.4, while the preservative solution had the smallest increase in pH of 0.5. The acidic solution electrical conductivity (EC) increased the most by 0.09 dS·m⁻¹, while the control and basic solution increased by 0.04 dS·m⁻¹, and the preservative had no change. The lowest percentage of petal blueing occurred in the preservative solution (59%) and was not significantly different between the other vase solutions (82%-94%). In the sterilized stems only, all vase solutions and all bacteria treatments there were significant differences in partial percent loss in conductivity (PPLC), which was lowest in the acidic solutions at 64% and significantly different from the preservative at 87% and the control at 83%. Adding the bacterium *E. coli* K 12 to vase solutions resulted in a comparable number of days to the first signs of water stress as a commercial holding solution even without sanitizing the cut stems. For species that are less affected by bacteria, this research provides insight into the use of low pH solutions for improving stem hydraulic conductivity. By combining the benefits of low pH, *E. coli* K 12, and other components, such as sugars, a novel organic floral preservative that prolongs vase life may be possible.

1. Introduction

Solution pH has been found to be an important factor in vase life for many species of cut flowers, with low pH (3.0-4.0) increasing the vase life of *Hydrangea* (Thunb.) Ser. (Ahmad et al., 2013), *Dendranthema* L. (Carlson and Dole, 2013), *Helianthus* L. (Carlson and Dole, 2013; Stevens et al., 1993), and *Rosa* L. (Conrado et al., 1980; Durkin, 1979; Regan and Dole, 2010). There are many hypotheses to explain the increase in vase life including: slowed bacterial growth in the vase
solution (Conrado et al., 1980; van Doorn and Perik, 1990), improved stem water uptake (Gast, 2000; Sacalis and Seals, 1993), and reduced embolization (Durkin, 1979). Marousky (1971) found that stems held in an acidic pH (3.0) had a higher hydraulic conductance than stems held in a more basic pH (6.0) and resulted in the same hydration effects as cutting stems under water, suggesting a reduction of emboli (Durkin, 1979).

A major cause of deterioration in cut flowers is blockage of xylem vessels by microorganisms that accumulate in the vase solution or in the vessels themselves (Knee, 2000) resulting in petal and leaf wilt, bent neck, tissue desiccation, or similar water stress related symptoms (Put, 1990; Put, 1986; van Doorn et al., 1991; Zagory and Reid, 1986; de Witte and van Doorn, 1988). However, the effects of bacteria concentrations in vase solutions differ with cut flower species. Put and Jansen (1989) found cut *Rosa* ‘Sonia’ vase life was reduced by bacteria concentrations as low as $10^5$ cfu·ml$^{-1}$, while Jones and Hill (1993) found cut *Dianthus caryophyllus* L. ‘Medea’, *Iris* L., *Alstroemeria* L., and *Tulipa* L. tolerated bacterial counts up to $10^8$ cfu·ml$^{-1}$. Therefore, the number of bacteria in the vase solution may not be the primary cause of wilting or shortened vase life in all cut flower species. Controlling the species of bacteria present in the vase solution may be more important than just the concentration. Some bacterial species produce exopolysaccharides that clog the vasculature (Put, 1990), enzymes that degrade plant tissue (Membre and Burlot, 1994), and hormones, like ethylene, that cause senescence (van Doorn, et al., 1991).

In addition, as the number of organic cut flower farms increases, the need for organic floral preservatives that effectively control microbial growth also increases. Commercial floral preservatives have three general components: an acidifier, a biocide, and a carbohydrate (sugar). Certified organic sources of the acidifier and carbohydrate are relatively easy to obtain, but a biocide
is difficult. Research on microbial growth in vase solutions has identified bacteria that have differing
effects on vase life: detrimental, neutral, and beneficial (Jacob and Kim, 2010; van Doorn et al.,
1991; Zagory and Reid, 1986, de Witte and van Doorn, 1988; Putt, 1986). Beneficial bacteria that
would improve vase life by preventing growth and proliferation of phytopathogens by producing
toxic metabolites, out-competing detrimental bacteria for nutrition and colonization sites, or
triggering defense response-related genes in plants (Gao et al., 2012) could be added as an organic
probiotic to vase solutions. The proposed beneficial bacterium is *Escherichia coli* K12, a non-human
pathogenic, laboratory adapted, Hfr Hayes strain from the Matthysse lab collection. The detrimental
strain used in these studies was *Pseudomonas marginalis*, a soil bacterium known to cause soft rots
in plant tissues (Li et al. 2007; Liao et al., 1997), isolated from cut *Zinnia* stems. The objective of this
research was to determine the effects of detrimental and beneficial bacteria, solution pH, and their
interactions on vase life, bacterial growth inside and outside the stem, and stem hydraulic
conductivity using cut *Zinnia* ‘Benary’s Giant Wine’.

2. Materials and methods

Cut stems of *Zinnia* ‘Benary’s Giant Wine’ (Harris Seeds, Rochester, NY, USA) were harvested
from field beds planted on 15 May 2013 in Raleigh, NC in 1.2 x 30.5 m, loamy clay soil beds. Stems
were harvested into tap water (6.1 pH, EC 0.21 dS·m⁻¹) and allowed to hydrate for one hour before
being placed into treatments. Stems were sorted into twenty-four groups of four stems each,
according to flower head size, and stems cut to 30 cm, labeled, and placed into treatments. Sixteen
groups of stems were surface sterilized by wiping the lower portion of the stems with sterile wipes
drenched in 70% ethanol before cutting with sterile clippers to the final length (30 cm), ensuring
that no ethanol came into contact with the cut surface, and placed into treatments. The remaining
eight groups were not surface sterilized. Treatments were arranged in a 4 vase solutions x 4 bacteria treatment factorial design for sterilized stems and a 4 x 2 factorial design for non-sterilized stems (24 total treatments), using the treatments described below.

2.1 Vase solutions

For both sterilized and non-sterilized stems, vases of 500 mL of sterilized deionized (DI) water were amended to a pH of 3.0 (EC 0.01 dS·m⁻¹) with 1N hydrochloric acid (HCl), 3.5 (EC 0.41 dS·m⁻¹) with a floral preservative holding solution (10 mL·L⁻¹; Floralife Professional, Walterboro, SC, USA), or 8.0 (EC 0.02 dS·m⁻¹) by adding 0.3M sodium hydroxide (NaOH). A sterilized DI water control (pH 5.2, EC 0.00 dS·m⁻¹) was also included. Glassware and solutions were sterilized via autoclaving.

2.2 Bacteria inoculations

The bacteria for inoculation were cultured for approximately 4 d at 21 ± 2 °C in 10 mL of nutrient broth (Remel, Thermo Fisher Scientific, Lenexa, KS, USA) on a shaker (Gyrotory water bath shaker, Model G76, New Brunswick Scientific Co., Inc., Edison, NJ, USA) to a concentration of approximately 10⁸ cfu·mL⁻¹, which was diluted to a final concentration of 10⁵ cfu·mL⁻¹ in the vases. Sterilized and non-sterilized stems had different sets of bacteria inoculation treatments. Sterilized stems were inoculated with either: 1) 0.5 mL beneficial bacteria, 2) 0.5 mL detrimental bacteria, 3) 0.25 mL beneficial + 0.25 mL detrimental bacteria combination, or 4) not inoculated. The non-sterilized stems were inoculated with 0.5 mL beneficial bacteria or not inoculated.

2.3 Experimental design

Each treatment had one vase with four stems per vase and was replicated six times over the course of a month (30 July to 30 August 2013). Vases were arranged in a complete randomized design and placed in a postharvest environment at 21 ± 2 °C at 40-60% relative humidity under
approximately 20 µmol·m⁻²·s⁻¹ light for 12 h·d⁻¹. Days to water stress (DTWS) was determined by observing stems daily and terminating them when initial signs of water stress such as petal wilt, desiccation, or bent neck started to show; symptoms were recorded for each stem as present or not present. Incidence of petal blueing was also recorded as present or not present at termination. One stem in each vase was weighed for initial fresh weight and again at termination and dried for final dry weight. When the last stem in each vase was terminated, the final solution pH and EC were recorded along with a visual solution turbidity rating on a scale from 1 to 4; with 1 being clear and 4 being very turbid.

2.4 Hydraulic conductivity

At termination, hydraulic conductivity of one stem in each vase was measured according to the methods of Sperry et al. (1988) to quantify stem blockage or percent loss of conductivity (PLC). Deionized water was used at a pressure of 5.88 kPa. Conductivity of the entire stem was measured minus the upper 6.5 cm (23.5 cm length) portion of the stem (the fragile hollow neck), which was removed to fit the apparatus tubing snugly to the stem. The bottom of the stem was not recut. If there was zero conductivity at the 23.5 cm length, the same stem was then re-measured after removing the lower 5 cm (18.5 cm final length) by cutting underwater. Ten stems were harvested directly from the field beds and measured for their hydraulic conductivities at both stem lengths (23.5 and 18.5 cm) for comparison to the treated stems. Fresh stems were recut to the appropriate length underwater before measuring. PLC was calculated as \( \text{PLC} = \frac{(k_f - k_t)}{k_f} \cdot 100 \), where \( k_f \) is the average fresh stem flow rates and \( k_t \) is the flow rate of a treated stem. Separate PLC calculations were performed for the 23.5 cm full stem length (FPLC) and the 18.5 cm partial stem length (PPLC).
2.5 Bacteria quantification and identification

At termination, using aseptic techniques, one stem in each vase had the lower 5 cm surface sterilized with 70% alcohol, cut from the stem and in half lengthwise, exposing the vasculature, and placed into a test tube. The test tube was pre-filled with 9.9 mL of sterilized deionized water and 0.25 g of quartz sand. The test tube was then sonicated for 2 min using an ultrasonic cleaner (Model FS20, Thermo Fisher Scientific, Lenexa, KS, USA) to dislodge bacteria from the inside of the stem. Also at termination, another stem in each vase had the lower 5 cm of the stem removed and placed into test tubes as described previously to collect bacteria from the outside of the stem. Bacteria from inside and outside the stem were quantified using serial dilutions up to $10^{-8}$ cfu·mL$^{-1}$ and plated onto nutrient agar media (Remel, Thermo Fisher Scientific, Lenexa, KS, USA) for counting. For one replication, samples collected from treatments inoculated with the beneficial bacteria were plated on MacConkey’s media (Remel, Thermo Fisher Scientific, Lenexa, KS, USA) and *E.coli* was identified by the characteristic pink dotted colonies. Samples collected from treatments inoculated with the detrimental bacteria were also plated on Mueller Hinton media (Remel, Thermo Fisher Scientific, Lenexa, KS, USA) and *P. marginalis* was identified by the characteristic yellow color produced by the colonies. All plates used during these studies contained 0.1% cyclohexamide to prevent fungal growth.

2.6 Statistical analysis

Data were analyzed using SAS/STAT software (Version 9.3, SAS Institute, Inc., Cary, NC). To appropriately balance the treatment levels the experiment was analyzed in two separate analyses. One analysis included beneficial and no bacteria treatments only, all vase solutions, and both sterile and non-sterile stems and the second included only sterilized stems, all vase solutions, and all
bacteria treatments. Data were analyzed as a randomized complete block design by Analysis of Variance (ANOVA) using the PROC MIXED procedure using vase as the experimental unit and replication as the block. Means were separated using the macro PDMIX800.sas (Saxton, 1998) and the output produced by the LSMEANS statement with the PDIF option and Tukey-Kramer adjustment at α = 0.05.

3. Results

3.1 Analysis of beneficial and no bacteria treatments only using all vase solutions and sterile and non-sterile stems

There was a significant three-way interaction for DTWS (P=0.0388) (Figure 1). The non-sterile stems/control solution/beneficial bacteria and non-sterile stems/preservative solution/no bacteria treatments had the most DTWS of 8.0 d. The sterile stems/control solution/beneficial bacteria and sterile stems/basic solution/no bacteria treatments had the lowest average DTWS of 5.5 d and 5.8 d, respectively.

The number of bacteria inside and outside the stem were significantly lower in the preservative solution compared to acidic, basic, and control solutions (P<0.0001), which were not significantly different from one another (Table 1). The vase solution pH of the acidic solution increased the most at 3.4, while the basic solution decreased by 1.2, and the preservative solution had the smallest increase in pH of 0.5 (P<0.0001). The acidic solution EC increased the most by 0.09 dS·m⁻¹, while the control and basic solution increased by 0.04 dS·m⁻¹, and the preservative solution showed no change (P<0.0001). The turbidity rating was lowest in the preservative solution (2.0 out of 4.0) and highest in the acidic solution (3.0) (P=0.0003). Petal blueing was significantly reduced in
the preservative solution (59%) \((P<0.0001)\) compared to the other vase solutions (82%-94%), which were similar.

3.2 Analysis of sterilized stems only using all vase solutions and all bacteria treatments

The same parameters were effected by vase solution as in the above analysis except in this set PPLC was also effected by the vase solutions (Table 2). Similar trends were observed as above. The preservative solution had a significantly lower bacteria concentration inside and outside the stem, the lowest turbidity rating, and the least percent incidence of petal blueing \((P<0.0001)\). PPLC was lowest in the acidic solutions at 64%, which was significantly different from the preservative at 87% and the control at 83%, the basic solution was not significantly different from any of the other solutions at 79% \((P=0.0041)\). The FPLC was not significantly affected by any of the treatments.

\(E. \ coli\) was detected on plates from all treatments in which it was inoculated both inside and outside the stem. \(P. \ marginalis\) was detected in all treatments inside and outside the stem except for the following: inside and outside the sterile stems/preservative solution/combination of bacteria and sterile stems/control solution/ detrimental bacteria stems, inside the sterile stems/acidic solution/detrimental bacteria stems, and outside the sterile stems/preservative solution/detrimental bacteria treatment.

The incidence of desiccated and wilted petals and change in stem fresh weight were not significantly affected by any of the treatments (data not presented). Interactions not mentioned above were not statistically significant.

4. Discussion

\(Zinnia\) is commonly referred to by growers as a “dirty flower” meaning it carries lots of bacteria with it from the field quickly dirtying the water the stems are held in. \(Zinnia\) stems have tiny
hairs along the stem that easily carry bacteria and provide them with a place to grow. This is also observed with the hairy stems of cut *Gerbera* where the number of bacteria in a vase solution containing one gerbera flower after one day ($10^6$ cfu·mL$^{-1}$) (van Doorn and deWitte, 1994) matched that of one rose flower after two to three days (de Witte and van Doorn, 1988). Put (1990) also found that the microbial loads on stems of cut *Rosa* were much lower than that on the stems of *Chrysanthemum* and *Gerbera*. *Zinnia* also produces cut flowers with water balance related vase life problems, where transpiration exceeds uptake causing tissue wilt and desiccation (Twumasi et al., 2005) making it a good candidate for examining the effects of vase solution pH and bacteria.

4.1 Effects of interactions between vase solution and bacteria

It was important to compare the pH and bacteria treatments to a commercial floral holding solution to see how a novel treatment would perform relative to the industry standard. The non-sterile stems/control solution/beneficial bacteria and non-sterile stems/preservative solution/no bacteria treatments had the same DTWS (8.0 d), which was the highest of all the treatments. This illustrates that there is significant potential for *E. coli* K 12 to be used as a probiotic in vase solutions. Positive results were seen without having to sterilize the stems and sterilizing stems might even be detrimental to the control/beneficial treatments as shown in the 2.5 d DTWS difference between the sterile and non-sterilized stems. Growers are unlikely to want to take the extra time and added expense of sterilizing stems. This may also indicate that the use of tap water rather than sterilized DI water may be acceptable, but this remains to be tested.

We chose to take data when stems started to show the first signs of water stress (DTWS) rather than at a typical vase life because the conditions causing the decline in stem quality would be more informative than when stems had already significantly senesced. To determine vase life, stems
are usually terminated when they show at least 50% of a termination criterion, such as petal wilt, desiccation, discoloration, or bent or collapsed stem. If data were taken when stems were terminated for vase life, stem tissue would have been too decomposed to determine the bacteria concentrations and hydraulic conductivities were causing the postharvest quality to decline. We hypothesize that PLC would have been close to 100% at vase life termination.

4.2 Effects of vase solution pH

Generally, differences were not significant between acidic (HCl), control, and basic treatments, which agrees with previous studies, showing that *Zinnia* was not significantly affected by solution pH (Carlson and Dole, 2013). Many of the significant effects observed with the preservative were likely due to the buffers it contains to maintain a consistently low pH, since similar results were not seen in the acidic (HCl) treatments. If the acidic solution had been buffered, we may have seen a reduction in bacteria counts because a low pH could have been maintained for longer to control growth more effectively. Not all bacteria are killed by low pH (Hosein et al., 2011), their growth may just be slowed or delayed, which would allow for subsequent proliferation if the pH was not kept constantly low. There are also bacteria species that prefer acidic environments that would not be negatively affected by acidic vase solutions. Preservatives not only contain compounds that reduce the pH, but also have long acting biocides that can account for the significant reduction in bacteria populations inside and outside the stems and the low turbidity rating. The preservative solution also had no change in solution EC, while the other solutions increased. The increase in solution EC could have a role in high numbers of bacteria in acidic, basic, and control solutions, as cell walls in the stem lysed and released cellular components, which bacteria can utilize for growth. Numbers of *P. marginalis* not *E. coli*, were significantly reduced by preservatives illustrated by the
consistent presence of *E. coli* and not *P. marginalis* in preservative treatments. This may be due to the particular biocide in the preservative or the pH. *E. coli* prefers a pH of 6-8, but can tolerate 4.4-9.0. *P. marginalis* also prefers a neutral pH of 7 (Membre and Burlot, 1994). A tolerable range for *P. marginalis* could not be found in the literature, but acid culture has been found to inhibit growth (Membre and Burlot, 1994).

Preservatives reduced the incidence of petal blueing in *Zinnia* likely due to the carbohydrates it contains. Sucrose and other carbohydrate-based preservatives have been found to reduce petal blueing in cut *Rosa hybrida* L. (Kesta and Dadaung, 2007; Marousky and Carlyle, 1985; Halevy and Mayak, 1979). A solution pH effect can be discounted since the acidic HCl solution did not similarly reduce blueing.

4.3 Effects of bacteria concentrations and species

Overall, there were no significant effects of bacteria or whether stems were sterilized or not. Vase solutions had such significant effects that it may have made it hard to clearly see how the bacteria were effecting postharvest. It is likely that we would see a difference between the bacteria in a simplified experiment that focused on the bacteria only.

Biocontrol bacteria usually confer their positive effects by preventing growth and proliferation of phytopathogens by producing toxic metabolites, out-competing for nutrition and colonization sites, and triggering defense response-related genes in plants (Gao et al., 2012). *Escherichia coli* K 12 is a debilitated non-pathogenic laboratory strain of *E. coli* that has a safe history of commercial use and no known adverse effects on plants (US Environmental Protection Agency, 1997). *E. coli* was positively identified on MacConkey media from final vase solutions in all beneficial and combination treatments, but not quantified. *P. marginalis* is a phytopathogenic bacterium that
produces pectinolytic enzymes that breakdown the middle lamella and cells walls of plants tissues (Membre and Burlot, 1994) and was identified from final vase solution treatments in many of the inoculated treatments.

While we did not quantify the number of bacteria in the vase solution, solution turbidity ratings paralleled well with bacteria counts inside and outside of stems. Van Doorn and deWitte (1991) also found that the number of bacteria in the vase solution highly correlated with the number of bacteria in the lower 5 cm of the stem. Turbidity could have also been related to and influenced by the change in EC with lysed cell contents clouding the water or from bacteria creating products like exopolysaccharides (EPS). The preservative with its long acting biocide allowed for fewer bacteria to proliferate and cloud the vase solution resulting in a low turbidity rating. However, a lower turbidity rating did not correlate with greater postharvest quality.

4.4 Effects on hydraulic conductivity

Differences in percent loss of conductivity (PLC) were only significant with the lower 5 cm removed for the partial stem measurements (PPLC). Many stems only conducted solution after the lower 5 cm were removed where blockages from bacteria and other microorganisms are likely to occur. Van Doorn et al. (1989) found that the basal 5 cm of the stems limited the flow of water and that this blockage was correlated with a high number of bacteria in this stem segment, which we also found. This data reinforces the benefits of recutting stems after a period of time to continue to facilitate uptake. Put and Jansen (1989) found that stem hydraulic conductivity was lowest in the basal 5 cm of the stem of cut Rosa and when two consecutive 5 cm stem segments were measured conductivity slightly increased as they went up the stem. PPLC was significantly different between the vase solution treatments in the sterilized stems/all vase solutions/all bacteria treatments. The
lowest PPLC was in the acidic solutions at 64%, which was significantly different from the preservative at 87% and the control at 83%; the basic solution was intermediate at 79%. Durkin (1979) hypothesized that the acidified water enhanced lateral water movement through vessel walls, possibly by breaking calcium pectate bonds. Marousky (1971) found that stems held in a pH of 3.0 had a higher hydraulic conductance than stems held in a pH of 6.0; no bacteria were found in the vase solution, so a bacterial effect was excluded. These differences in PPLC show some agreement with the theory that acidic vase solutions help to reduce stem blockage. There were no significant differences in bacteria counts inside and outside the stem and the turbidity rating so a bacterial effect is not likely. This same lack of correlation between high bacteria counts and less of a reduction in PLC was also seen by Williamson and Milburn (1995) where stems held in citric acid showed less xylem cavitation and higher bacteria counts in the vase solution. They found no clear relationship between cavitation events and the number of bacteria in the vase solution and hypothesized that citric acid was digesting pit membranes (Williamson and Milburn, 1995).

5. Conclusion

Adding the bacterium *E. coli* K 12 to vase solutions resulted in a comparable number of days to the first signs of water stress as a commercial holding solution even without sanitizing the cut stems. These studies have the potential to provide cut flower growers, suppliers, and consumers with organic methods for controlling bacterial growth in postharvest solutions. Other cut flower species need to be tested to see if the beneficial bacteria would have a positive effect on more than just *Zinnia* as the effects of the bacteria are likely to be species dependent. For species that are less affected by bacteria, this research provided insight into the use of low pH solutions for improving hydraulic conductivity in the stem. By combining the benefits of low pH, *E. coli* K 12, and other
components to be determined, such as sugars and other nutrients, a novel organic floral
preservative that prolongs vase life may be possible.

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Blair Lane. We would also like to thank Joy Smith for statistical consulting.
References


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<http://epa.gov/biotech_rule/pubs/fra/fra004.htm>


Tables

Table 1. Analysis of beneficial and no bacteria treatments only, using all vase solutions and sterile and non-sterile stems. Effects of vase solution on bacterial concentrations inside and outside the stem, change in vase solution pH and EC, final vase solution turbidity rating (scale from 1 to 4; 1 being clear and 4 being very turbid), and percent incidence of petal blueing. Basic solutions were altered with NaOH (pH 8.0, EC 0.02 dS·m⁻¹), acidic solutions with HCl (pH 3.0, EC 0.01 dS·m⁻¹), preservative was Floralife professional (pH of 3.5, 0.41 dS·m⁻¹), and the control was sterile deionized water (pH 5.2, EC 0.00 dS·m⁻¹). Means were an average of 24 stems except blueing, which was an average of 96 stems.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Inside (cfu·mL⁻¹)</th>
<th>Outside (cfu·mL⁻¹)</th>
<th>Change in pH</th>
<th>Change in EC (dS·m⁻¹)</th>
<th>Turbidity rating</th>
<th>Blueing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic</td>
<td>6.89 x10⁹ a&lt;sup&gt;y&lt;/sup&gt;</td>
<td>6.15 x10⁹ a&lt;sup&gt;y&lt;/sup&gt;</td>
<td>3.4 a</td>
<td>0.09 a</td>
<td>3.0 a</td>
<td>94 a</td>
</tr>
<tr>
<td>Control</td>
<td>1.00 x10¹⁰ a</td>
<td>5.71 x10⁹ a</td>
<td>1.5 b</td>
<td>0.04 b</td>
<td>2.6 a</td>
<td>91 a</td>
</tr>
<tr>
<td>Basic</td>
<td>4.89 x10⁹ a</td>
<td>9.21 x10⁹ a</td>
<td>-1.2 d</td>
<td>0.04 b</td>
<td>2.5 ab</td>
<td>82 a</td>
</tr>
<tr>
<td>Preservative</td>
<td>1.46 x10⁹ b</td>
<td>8.84 x10⁸ b</td>
<td>0.5 c</td>
<td>0.00 c</td>
<td>2.0 b</td>
<td>59 b</td>
</tr>
<tr>
<td>Significance&lt;sup&gt;x&lt;/sup&gt;</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0003</td>
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</table>

<sup>y</sup>Means followed by the same letter are not significantly different according to the macro PDMIX800.sas (Saxton, 1998) and the output produced by the LSMEANS statement with the PDIFF option and Tukey-Kramer adjustment at α = 0.05

<sup>x</sup>P values were obtained by PROC MIXED procedures using SAS/STAT software (Version 9.3, SAS Institute, Inc., Cary, NC)
Table 2. Analysis of sterilized stems only, using all vase solutions and all bacteria treatments. Effects of vase solution on partial stem percent loss of conductivity (PPLC), bacterial concentrations inside and outside the stem, change in vase solution pH and EC, final vase solution turbidity rating (scale from 1 to 4; 1 being clear and 4 being very turbid), and percent incidence of petal blueing. Basic solutions were altered with NaOH (pH 8.0, EC 0.02 dS·m⁻¹), acidic solutions with HCl (pH 3.0, EC 0.01 dS·m⁻¹), preservative was Floralife professional (pH of 3.5, 0.41 dS·m⁻¹), and the control was sterile deionized water (pH 5.2, EC 0.00 dS·m⁻¹). Means were an average of 24 stems except blueing, which was an average of 96 stems.

<table>
<thead>
<tr>
<th>Solution</th>
<th>PPLC (%)</th>
<th>Inside (cfu·mL⁻¹)</th>
<th>Outside (cfu·mL⁻¹)</th>
<th>Change in pH</th>
<th>Change in EC (dS·m⁻¹)</th>
<th>Turbidity rating</th>
<th>Blueing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic</td>
<td>64 b</td>
<td>1.52 x10¹⁰ a</td>
<td>1.34 x10¹⁰ a</td>
<td>3.4 a</td>
<td>0.09 a</td>
<td>3.1 a</td>
<td>88 a</td>
</tr>
<tr>
<td>Control</td>
<td>83 a</td>
<td>8.87 x10⁸ a</td>
<td>5.91 x10⁷ a</td>
<td>1.5 b</td>
<td>0.04 b</td>
<td>2.7 a</td>
<td>91 a</td>
</tr>
<tr>
<td>Basic</td>
<td>79 ab</td>
<td>7.29 x10⁹ a</td>
<td>9.78 x10⁸ a</td>
<td>-1.2 d</td>
<td>0.03 b</td>
<td>2.6 a</td>
<td>82 a</td>
</tr>
<tr>
<td>Preservative</td>
<td>87 a</td>
<td>1.15 x10⁹ b</td>
<td>1.14 x10⁹ b</td>
<td>0.5 c</td>
<td>0.00 c</td>
<td>1.7 b</td>
<td>54 b</td>
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<tr>
<td>Significance</td>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

xMeans followed by the same letter are not significantly different according to the macro PDMIX800.sas (Saxton, 1998) and the output produced by the LSMEANS statement with the PDIFF option and Tukey-Kramer adjustment at α = 0.05

P values were obtained by PROC MIXED procedures using SAS/STAT software (Version 9.3, SAS Institute, Inc., Cary, NC)
Figure 1. Analysis of beneficial and no bacteria treatments only, using all vase solutions and sterile and non-sterile stems. The effects of sterilizing stems, vase solution, and bacteria inoculations ($10^5$ cfu·mL$^{-1}$) on days to first sign of water stress (DTWS). Basic solutions were altered with NaOH (pH 8.0, EC 0.02 dS·m$^{-1}$), acidic solutions with HCl (pH 3.0, EC 0.01 dS·m$^{-1}$), preservative was Floralife professional (pH of 3.5, 0.41 dS·m$^{-1}$), and the control was sterile deionized water (pH 5.2, EC 0.00 dS·m$^{-1}$). Means were an average of 24 stems; $P=0.0388$. Error bars represent the 95% confidence interval.
CH. 4: USING RNA-SEQ TECHNOLOGY AND DIFFERENTIAL EXPRESSION ANALYSIS TO ELUCIDATE THE POTENTIAL CAUSES OF PEDUNCLE BENDING AND PETAL BLUEING IN CUT ROSA ‘FREEDOM’ AND ‘FOREVER YOUNG’

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Abstract

An analysis of the changes in global gene expression patterns during the onset of peduncle bending (bent neck) and petal blueing in cut Rosa hybrida L. ‘Freedom’ has identified 297 and 337 differentially expressed genes compared to healthy tissues. ‘Forever Young’ averages a longer vase life and time to bent neck than ‘Freedom’ so differential expression analysis was also performed comparing healthy neck tissues of the rose two cultivars, resulting in 818 differentially expressed genes. The data suggest that bent neck may mostly be due to cell wall degradation of the peduncle related to general senescence processes and petal blueing may be due to sugar deprivation. ‘Forever Young’ may have a longer vase life because of the higher expression of peroxidase compared to ‘Freedom’. All samples had significant expression of genes related to various stressors including wounding, pathogens, and water deprivation. This work will provide further assistance to postharvest scientists creating novel vase solutions, breeders using marker assisted selections, and molecular geneticists transforming rose.
1. Introduction

*Rosa hybrida* L. continues to be one of the most important ornamental crops in the world. In 2012, the total wholesale value of cut roses in the United States was $16.8 million (USDA, 2013). The import value is even greater, at $367.3 million in 2012 (Global Agricultural Trading System, USDA).

Cut roses have postharvest issues that can reduce their vase life, which is an important factor in consumer perception of value. The most common postharvest problems for cut roses are bent neck and petal blueing (Zieslin, 1989). Bent neck may be related to water relations problems from a lack of water uptake caused by bacteria occlusions, air embolism, or physiological blockages caused by the stems themselves. Petal blueing is hypothesized to be caused by an increase in the vacuolar pH causing the anthocyanins to change colors (Marousky and Carlyle, 1985; Asen et al., 1971). Both disorders generally occur before the petals visibly wilt beyond levels that would be unacceptable to consumers. Commercial postharvest solutions attempt to ameliorate the problems by including a biocide, lowering the pH, and providing stems with carbohydrates. However, a more effective approach may come from understanding the gene expression changes during the onset of these symptoms, which would be especially useful in determining the cause of physiological blockages that are currently unexplained. Little is known about the molecular mechanisms that control these symptoms in rose. Such information could be used in the future to develop novel postharvest treatments, help breeders with marker-assisted selections, or genetically modify plants to prevent these symptoms and improve postharvest quality of roses.

RNA-seq has become the technology of choice for transcriptome investigation (Deng et al., 2011) allowing molecular geneticists to explore transcriptomes of nearly any species in many different ways without the need for prior genomic knowledge (Ekblom and Galindo, 2011). Thus,
this technique is particularly useful in these studies as the rose genome has not been completely sequenced. RNA-seq is more accurate and captures a broader range of expression levels compared to microarrays, because it does not rely on hybridization (Marioni et al, 2008; Fu et al., 2009).

The objectives of this research are to identify differentially expressed genes that occur in: 1) cut Rosa ‘Freedom’ during bent neck and petal blueing compared to healthy tissues to find factors that may play a role in the cause of these postharvest symptoms and 2) healthy neck tissue of two cultivars, ‘Freedom’ and ‘Forever Young’ to find possible cultivar differences causing ‘Forever Young’ to last longer than ‘Freedom’. This type of study is necessary to develop a deeper understanding of what genes are differentially expressed as the cut flower develops bent neck and petal blueing.

2. Materials and methods

2.1 Plant material and tissue collection

Cut Rosa ‘Forever Young’ stems were received on 26 February 2013 and ‘Freedom’ was received on 13 June 2013 from a commercial supplier (Colombia, South America). Upon receipt, stems were cut to 45 cm and the foliage removed leaving only the first set of uppermost leaves. Stems were placed three per jar of 400 mL deionized (DI) water (pH 5.2, electrical conductivity (EC) 0.00 dS·m⁻¹) in a postharvest environment at 21 ± 2 °C at 40-60% relative humidity under approximately 20 mol·m⁻²·s⁻¹ light for 12 h·d⁻¹.

Healthy tissue samples, ten peduncles (‘Freedom’ and ‘Forever Young’) and ten petals (‘Freedom’ only) were harvested 24 hours after the experiment began. The samples were immediately snap-frozen in liquid nitrogen. The remaining stems were monitored for the appearance of peduncle bending (bent neck) and petal blueing. Bent neck samples were collected when the peduncles showed initial loss of rigidity and stiffness and blue petals were collected as the
first sign of blue-purple coloration (Fig. 1). The collected materials were immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

2.2 RNA extraction, cDNA library construction, and sequencing

Total RNA from peduncles and petals from the aforementioned stages was extracted using a Spectrum™ plant total RNA kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Contaminating DNA was removed with an On-Column DNase Digest Kit (Sigma-Aldrich, St. Louis, MO, USA). The integrity of the extracted RNA was examined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany) according to the manufacturer’s instructions. One biological replicate was produced for each tissue sample by pooling samples from three different stems.

A cDNA library for each tissue sample was made using the Illumina TruSeq RNA v2 Library Prep Kit (Illumina, Inc., San Diego, CA, US) following the manufacturer’s instructions. rRNA was depleted during library preparation using oligo-dT beads to select for poly-adenylated mRNA. This method also excluded bacterial information because bacteria transcripts are not poly-adenylated. The cDNA library samples were sequenced on Illumina HiSeq 2500 (Illumina, Inc., San Diego, CA, US) sequencing platform and all preparation and sequencing steps were conducted according to the manufacturer's procedures. The cDNA samples were end-repaired and adapters added. The prepared samples were put into the flow cell and placed into the cBot cluster generation system. After cluster generation, the flow cell was placed in the Illumina® HiSeq 2500 system for sequencing as a 100bp single read run. Sequencing results were obtained through the standard data collection and analysis software available for the sequencing system.
2.3 Data analysis

Three assemblies were created from the RNA-seq data to represent expressed transcriptomes for ‘Freedom’, ‘Forever Young’, and for the combined data (‘Freedom’ + ‘Forever Young’). The assemblies were created de novo with the program Trinity (Grabher et al., 2011), which produced a set of transcripts. These transcripts can be compared to isoforms of a gene where there may be many “transcripts” per “component”. Expression analysis was performed with the Tophat and Cufflinks software suite (Trapnell et al., 2012). Assemblies were used as reference sequences and reads were mapped back to the assemblies using Tophat2 (v2.0.10) as needed for each of the following comparisons: 1) ‘Freedom’ bent neck vs. ‘Freedom’ healthy neck (‘Freedom’ reference), 2) ‘Freedom’ blue petal vs. ‘Freedom’ healthy petal (‘Freedom’ reference), and 3) ‘Freedom’ healthy neck vs. ‘Forever Young’ healthy neck (combined reference). More than 75% of all reads in each dataset mapped to the references. The program Cufflinks (v2.1.1) was used to create a listing of transcripts based on the mappings and Cuffmerge (v2.1.1) established a final transcriptome for use in each of the three comparisons listed above. With the read mappings and the merged transcriptome assembly annotations, the program Cuffdiff (v2.1.1) was able to get count data for each transcript and normalize the counts to take a log-ratio measurement to determine differential expression. Cuffdiff used a geometric normalization method where fragments per kilobase of exon per million fragments mapped (fpkm) and fragment counts are scaled via the median of the geometric means of fragment counts across all libraries as described by Anders and Huber (2010). Significance values were adjusted for multiple testing, and results were reported for each experiment with an adjusted p-value of 0.05. The resulting differentially expressed sequences were annotated by BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990) and subsequently
mapped and categorized by gene ontology with Blast2GO (Conesa et al., 2005) using program default settings. Fold changes in fpkm were calculated by dividing bent neck expression by healthy neck expression, blue petal expression by healthy petal expression, and ‘Freedom’ healthy neck expression by ‘Forever Young’ healthy neck expression. Where there was zero expression for a particular differentially expressed gene the fpkm expression is given for the tissue in which it was expressed.

3. Results and discussion


3.1 ‘Freedom’ Bent Neck vs. ‘Freedom’ Healthy Neck

This comparison had 297 differentially expressed transcripts. There were more genes being upregulated in bent neck tissue than healthy neck tissue. The majority of sequences matched with *Fragaria vesca* (Rosaceae), *Prunus persica* (Rosaceae), and *Vitis vinifera* (Vitaceae) sequences. When categorized by gene ontology biological processes, the majority of the genes expressed in this comparison were related to metabolic, cellular, and single-organism processes and responses to stimuli (Fig. 2). The metabolic processes corresponded to more specific processes, such as carbohydrate metabolism, proteolysis, oxidation reduction, and ethylene metabolism. Responses to stimulus included genes related to fructose stimulus, hormones [auxin, jasmonic acid (JA), and
abscisic acid (ABA), osmotic stress, defense and pathogen response, wounding, and water deprivation.

Many genes with greater expression in healthy neck tissue compared to bent neck tissue where related to wounding and pathogen responses and water transport, included chitinase-2 (0.01 fpkm fold change), xyloglucan endotransglucosylate/hydrolase (0.02 fpkm fold change), proline-rich cell wall protein (0.03 fpkm fold change), and plasma membrane aquaporin (0.05 log_{10} fpkm fold change) (Table 1). Chitinase-2, xyloglucan endotransglucosylate, and proline-rich cell wall proteins are all related to wounding and pathogen related responses (The UniProt Consortium, 2014). Proline-rich proteins are typically cell wall structural proteins (Salts et al., 1991). Xyloglucan endotransglucosylase can catalyze transglycosylation reactions that might integrate newly synthesized xyloglucans into the cell wall (Tiaz and Zeiger, 1998). In tobacco stems, proline-rich cell wall proteins became rapidly insolubilized upon wounding, and this cross-linking is associated with an oxidative burst that may directly attack pathogens and with a mechanical stiffening of the cell walls that may indirectly deter subsequent invasion by pathogens by causing a rapid cross-linking of phenolic compounds in the cell wall (Tiaz and Zeiger, 1998). It is likely that the cut rose stem is responding to the stress of being cut from the plant. Proline-rich cell wall proteins have also been found to be expressed more in the young fruit tissue of tomato compared to mature tissue (Salts et al., 1991). Aquaporins are water-selective channel proteins whose expression can be controlled by osmotic stress and alter the rate of permeability (Tiaz and Zeigler, 1998). The high expression of these proteins in healthy tissue likely means that there is more water available to cross membranes to where it is needed in healthy tissues. As water becomes more limited over the life of the flower
and water uptake slows, the need for aquaporins decreases and the membranes become less permeable.

Many of the genes that were more highly expressed in bent neck tissues were related to senescence and programmed cell death, including vignain-like (292 fpkm fold change), β-galactosidase (97 fpkm fold change), stay green 1 (52 fold change), and β-xylosidase (26 fpkm fold change). Vignain-like protein is involved with programmed cell death (PCD) and proteolysis (The UniProt Consortium, 2014) and can be induced by osmotic stress (Tiaz and Zeigler, 1998). Stay green proteins are known to have a major role in chlorophyll and photosynthetic pigments degradation and are commonly associated with the processes of fruit ripening and organ senescence (Dubois et al., 2011). β-galactosidase and β-xylosidase are associated with softening of ripening strawberry fruits by degrading galactose and xylose, components of the cell wall (Trainotti et al., 2001; Martinez et al., 2004). β-xylosidase was found in strawberry fruit during ripening and was repressed by auxin (Trainotti et al., 2001; Martinez et al., 2004). In these studies we found more auxin expression related genes in healthy neck tissue than bent neck tissue. We found higher expression of JA and ABA induced genes in bent neck tissues. Bustamante et al. (2009) found that β-xylosidase could also be induced by ABA. Therefore, β-galactosidase and β-xylosidase might contribute to bent neck in rose by accelerating peduncle cell wall degradation. Zhu (2010) also found an increase in β-galactosidase and β-xylosidase expression in the bent neck tissue of ‘Freedom’. Laccase (138.9 fpkm) was only expressed in bent neck tissue and can be induced by ethylene (Ahmadi et al., 2009). Zhu (2010) also found high expression of laccase in ‘Freedom’ bent neck samples. Zhu (2010) also found an increase in expression of trehalose-6-phosphate
synthase/phosphatase, an indicator of water stress, in the bent necks of ‘Freedom’ roses; however, we did not find this protein being expressed in significant levels in our samples.

3.2 ‘Freedom’ Blue Petal vs. ‘Freedom’ Healthy Petal

‘Freedom’ was more susceptible to blue petals than ‘Forever Young’. ‘Forever Young’ petals tended to turn black very quickly during postharvest rather than blue so tissue was not collected because RNA was assumed to be too severely degraded. This comparison for ‘Freedom’ had 337 differentially expressed transcripts. There were more genes being downregulated in blue petal tissue than healthy petal tissue. The majority of sequences matched with _Fragaria vesca_ (Rosaceae), _Prunus persica_ (Rosaceae), and _Vitis vinifera_ (Vitaceae) sequences. When categorized by gene ontology biological processes, the majority of the genes expressed in this comparison were related to metabolic, cellular, and single-organism processes, and responses to stimulus (Fig. 3). The metabolic processes corresponded to more specific processes, such as carbohydrate metabolism, proteolysis, cell wall organization, and tissue development. Responses to stimulus included genes related to general stress, hormones, abiotic and biotic stimulus, and defense responses.

Several genes with greater expression in healthy petal tissue compared to blue petal tissue where related to wounding/pathogen responses, ethylene synthesis, and anthocyanin biosynthesis, included plasma membrane intrinsic protein isoform 1 (0.0003 fpkm fold change), repetitive proline-rich cell wall protein 2 (0.010 fpkm fold change), expansin 3 (0.003 fpkm fold change), xyloglucan endotransglycosylase (0.007 fpkm fold change), and chalcone synthase (0.009 fpkm fold change) (Table 2). We found upregulation of xyloglucan endotransglucosylase and proline-rich cell wall proteins, related to wounding and pathogen related responses (Tiaz and Zeigler, 1998), similar to those found in healthy necks in the first comparison above. Xyloglucan endotransglycosylase were
shown to be induced in senescing petals by ethylene (Singh et al., 2011). The plasma membrane intrinsic protein isoform 1 may be related to brassinosteroid biosynthetic process and the acetyl-CoA metabolic process (The UniProt Consortium, 2014). Expansin is a wall loosening protein that induces stress relaxation of the wall by loosening hydrogen bonds between hemicellulose and microfibrils (Tiaz and Zeigler, 1998). Expansin 1A was found to be specifically induced during petal abscission (Singh et al., 2011). There was also an increased expression of chalcone synthase and udp-glycosyltransferase 85a3-like (3-O-glucosyltransferase), both involved in flavonoid biosynthesis (The UniProt Consortium, 2014). Expression of chalcone synthase is enhanced by sugar abundance (Koch, 1996) and is the initial step for flavonoid biosynthesis. Plant pigments, pelargonidin and cyanidin are glucosylated by 3-O-glucosyltransferase to become the more stable pelargonidin 3-glucoside and cyanindin 3-glucoside, respectively. Anthocyanin biosynthesis is significantly downregulated in blue petal tissue, which could attribute to the change in coloration.

Many of the genes that were more highly expressed in blue petal tissue were related to pathogen/wound responses, sugar depletion, and cell wall softening, included class i chitinase (1367 fpkm fold change), asparaginase (37 fpkm fold change), proline dehydrogenase (118 fpkm fold change), and polygalacturonase (100.6 fpkm). Chitinase is a pathogen defense enzyme released by the plant to attack fungal walls, which are primarily composed of chitin (Tiaz and Zeigler, 1998). The increased expression of this enzyme may indicate Botrytis infection of the petals. Botrytis is a common pathogen on cut roses, which can significantly reduce postharvest quality. The initial signs of botrytis infection were observed on the petals of some of the flowers used in these studies (personal observation). While this defense response was highly expressed in blue petal tissues, it is not likely to have been a cause of blue petals. Proline dehydrogenase is activated in plants
recovering from abiotic stresses associated with water deprivation and is also a defense component contributing to hypersensitive response (HR) and disease resistance (Cecchini, et al., 2011). Cecchini et al. (2011) found increased proline dehydrogenase activity in cells destined to die and dependence on salicylic acid. Asparaginase expression is enhanced by sugar depletion (Koch, 1996) and could indicate that carbohydrates are a major limiting factor in rose postharvest life. Polygalacturonase (1,4-α-galacturonidase) is part of the carbohydrate metabolism pathway, converting pectate into D-galacturonate, which connects with ascorbate metabolism pathways.

Petal blueing in ‘Freedom’ could be caused by downregulation of anthocyanin biosynthesis related genes due to the lack of available sugars. Sucrose and other carbohydrate-based preservatives reduce petal blueing and prolong cut rose vase life (Kesta and Dadaung, 2007; Marousky and Carlyle, 1985; Halevy and Mayak, 1979). The major pigment in red rose petals is the anthocyanin, cyanidin 3,5-diglucoside (Lindstrom and Markakis, 1963). Asen et al. (1971) found petal blueing in rose was due to the co-pigment complex of cyanidin 3,5-diglucoside, quercetin, and kampferol glycosides. As petals aged, the pH increased from an increase in free cellular ammonia due to proteolysis (Marousky and Carlyle, 1985) resulting in petal blueing (Asen et al., 1971). Stoltz (1956) showed petals did not blue or become deficient in carbohydrates when preservatives containing glucose or sucrose were used and hypothesized that protein hydrolysis occurred only under conditions of carbohydrate starvation. Borochov et al. (2006) found that when added to the vase solution, sucrose retarded and ABA promoted processes associated with senescence, including petal wilt, increased pH in petal tissue, petal blueing, and a decrease in protein content of petals. They suggested that ABA accelerates senescence of cut roses by promoting petal growth and respiration, thus decreasing the carbohydrate level in the petals and triggering the chain of
metabolic processes leading to aging. ABA is highly expressed in senescent petals of ethylene sensitive and insensitive species and its action in rose senescencing petals is unclear (Bendahmane et al., 2013). Senescing rose petals have also been found to have increased indogenous H₂O₂ levels and decreased antioxidant enzyme activity (Kumar et al., 2008). Ethylene promotes petal abscission through cell wall modifications in rose (Bendahmane et al., 2013).

3.3 ‘Freedom’ Healthy Neck vs. ‘Forever Young’ Healthy Neck

Comparing the healthy necks of the two cultivars will help us to understand the differences in the cultivars that may be causing them to differentially susceptible to bent neck. This comparison had 818 differentially expressed transcripts. There were similar numbers of genes that are expressed in one cultivar and not the other. Only 24 had positive expression values for both cultivars. The majority of sequences matched with Fragaria vesca (Rosaceae), Prunus persica (Rosaceae), and Vitis vinifera (Vitaceae) sequences. Several of the most highly expressed transcripts in ‘Forever Young’ were uncharacterized proteins or had no BLAST hits (Table 3). Most sequences were metabolic or cellular processes related to binding and catalytic activity, including transferase and hydrolase activity and protein, lipid, and carbohydrate binding (Fig. 4). In general, the most of the genes were regulatory in nature or defense and stress response related.

Several genes had greater expression in healthy ‘Freedom’ tissue compared to healthy ‘Forever Young’ tissue were related to cell wall components, stress responses, and disease resistance (Table 3). Inositol oxygenase (20 fpkm fold change) was expressed more in ‘Freedom’ and is involved in the biosynthesis of UDP-glucouronic acid, providing nucleotide sugars for cell wall polymers and may also be involved in plant ascorbate biosynthesis (The UniProt Consortium, 2014). Heat shock proteins (24.4 fpkm) are encoded by common stress resistance related genes and help
plants survive under abiotic stresses and are found in many plant species (Vierling, 1991). Monothiol glutaredoxin (57.3 fpkm) has several possible roles including, auxin transport, oxidation-reduction processes, and response to heat (The UniProt Consortium, 2014). Again, we saw increased expression of proline-rich genes (115.2 fpkm). There was also a disease resistance protein rga3 that was only expressed in ‘Freedom’ at 317.6 fpkm.

Many of the genes that were more highly expressed in healthy ‘Forever Young’ necks were also related to cell wall components, stress responses, and disease resistance. The stress response protein NhaX had very high expression levels (0.05 fpkm fold change) and was found to be induced by phosphate starvation (The UniProt Consortium, 2014). A defense response related protein, MLP-like protein 28, was also expressed significantly more in ‘Forever Young’ (0.02 fpkm fold change). Peroxidase also had a high fold change (0.06 fpkm fold change) and could be involved in the removal of H₂O₂, oxidation of toxic reductants, in response to environmental stresses such as wounding, pathogen attacks, and oxidative stress (The UniProt Consortium, 2014). The much higher expression of this enzyme could contribute to the longer postharvest life of ‘Forever Young’ as compared to ‘Freedom’. There was 0.09 fpkm fold change expression of xyloglucan endotransglucosylate/hydrolase in ‘Forever Young’, which was also identified in the previous two comparisons. UDP-glycosyltransferase was highly expressed (0.07 fpkm fold change) and was also found in healthy petal tissue. GDSL esterase/lipase APG (0.01 fpkm fold change) is involved in lipid metabolism (The UniProt Consortium, 2014).

Several potential factors could contribute to ‘Forever Young’ having a longer postharvest life and less susceptibility to bent neck than ‘Freedom’. ‘Forever Young’ may have an enhanced ability to utilize carbohydrates and other nutrients and have more defense and protective mechanisms to
protect it from stressors. The uncharacterized and unknown sequences that have very high expression levels may play an important role in the extended vase life of ‘Forever Young’ and would be very informative if they could be identified further.

4. Conclusion

Bent neck may mostly be due to cell wall degradation of the peduncle related to general senescence processes. The expression of genes induced by pathogens and wounding may also play a significant role. Controlling plant pathogens like bacteria and fungi during postharvest handling might improve vase life by reducing the amount of energy plants have to spend on defense mechanism metabolism. A postharvest dip that could prevent wounding response induction would be ideal, as these responses likely trigger other stress responses that involve carbohydrate usage, programmed cell death, and cell wall degradation. Petal blueing may be due to sugar deprivation. Postharvest solutions containing high sugar content in forms readily available to the stems should be used. Antioxidants included in vase solutions could help to maintain processes going on in the stem to better utilize nutrients, sugars, and amino acids to maintain the stems health longer. While these studies provided a lot of information about the factors involved in bent neck and blueing in cut rose the number of identified sequences and lack of rose specific sequences was a limiting factor. This work will provide much assistance to postharvest scientists, breeders, and molecular geneticists, especially if the rose genome is sequenced in the future.

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Floriculture crops 2012 summary.


Table 1. Selected differentially expressed sequences with BLAST annotations from 'Freedom' bent neck vs. 'Freedom' healthy neck comparison.

<table>
<thead>
<tr>
<th>Bent neck (fpkm)</th>
<th>Healthy neck (fpkm)</th>
<th>Fold change (fpkm)</th>
<th>BLAST Sequence description</th>
<th>P-value</th>
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Table 2. Selected differentially expressed sequences with BLAST annotations from ‘Freedom’ blue petal vs. ‘Freedom’ healthy petal comparison.

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Table 3. Selected differentially expressed sequences with BLAST annotations from 'Freedom' vs. 'Forever Young' healthy necks comparison.

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<th>Forever Young (fpkm)</th>
<th>Fold change (fpkm)</th>
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<th>p-value</th>
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Figure 1a-b. The stage at which bent neck tissue samples (1a) and blue petal tissue samples (1b) were collected and snap-frozen for RNA extraction.
Figure 2. Total expression gene ontology by biological process (Level 2) from Blast2GO for ‘Freedom’ bent neck vs. ‘Freedom’ healthy neck.
Figure 3. Total expression gene ontology by biological process (Level 2) from Blast2GO for ‘Freedom’ blue petal vs. ‘Freedom’ healthy petal.
Figure 4. Total expression gene ontology by biological process (Level 2) from Blast2GO for ‘Freedom’ healthy neck vs. ‘Forever Young’ healthy neck.