ABSTRACT

Title of Thesis: Evaluation of Raspberry (*Rubus* sp.) Genotypes for Postharvest Quality and Resistance to *Botrytis cinerea*

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Raspberries are a delicate, high value specialty crop with an extremely short shelf life. This is exacerbated by their susceptibility to postharvest decay caused by *Botrytis cinerea*. Of the commercially available species, red raspberry (*Rubus idaeus* L.) is the most widely grown. Yellow (*R. idaeus L.*), black (*R. occidentalis* L.) and purple raspberries (*R.* × *neglectus* Peck.) are mainly available from direct marketers. The storageability of 17 cultivars was examined weekly from June to September during two growing seasons. Storage life was assessed weekly, while firmness, color, respiration and ethylene evolution rates were measured in select harvests. Black and purple raspberries outperformed red and yellow cultivars in their ability to resist *B. cinerea* colonization. Black raspberries also had the lowest ethylene evolution rates and incidence of decay. This information will be useful to raspberry breeding programs by identifying physiological characteristics that are correlated with greater resistance to *B. cinerea*.

EVALUATION OF RASPBERRY (*RUBUS* SP.) GENOTYPES FOR POSTHARVEST QUALITY AND RESISTANCE TO *BOTRYTIS CINEREA*

by

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List of Abbreviations

ACC 1-aminocylopropane-1-carboxylic acid

ACO ACC oxidase

ACS ACC synthase

AVG Aminoethoxyvinylglycine

DNA Deoxyribonucelic acid

ETR1 Ethylene receptor 1

ERS1 Ethylene response sensor 1

ETR2 Ethylene receptor 2

EIN4 Ethylene Insensitive 4

ERS2 Ethylene response sensor 2

GC Gas chromatography

ITS1 Intertranscribed ribosomal RNA spacer 1

ITS2 Intertranscribed ribosomal RNA spacer 2

KMBA 2-keto-4-methylthiobutryic acid

LS Means: Least squares means

ORAC Oxygen radical absorbance capacity

PCR Polymerase chain reaction

PDA Potato -dextrose agar

PDB Potato -dextrose broth

SAM S-adenosyl-methionine

SSC Soluble solids content

TA Titratable acidity

A Note to the Reader

Before you begin reading this thesis, I would like to familiarize you with its organization and structure. The organization is as follows: Chapter one contains an extended review of the pertinent literature. Chapter two is formatted as a manuscript to be submitted for review and potential publication upon completion of my Master's degree. Chapter three contains further discussions about my results, and things that I have learned about the scientific process during the course of this project, what I would change if I were to do it again and future studies. Following the three chapters are appendices that contain further results, and supplementary information. Enjoy!

Chapter 1: Literature Review

1.1. Taxonomy

Raspberries are a member of the rose family *(Roseacea)* in the genus *Rubus*. *Rubus* contains 14 subgenera and over 400 species. Raspberries are in the subgenus *Idaeobatus*, which contains the European red raspberry (*R. idaeus* subsp. *vulgatus* Arrhen or *R. idaeus* L.), the North American red raspberry (*R. ideaus* subsp. *strigosus* Michx or *R. strigosus* Michx), Eastern North American black raspberry (*R. occidentalis* L.) and purple raspberries which are hybrids between red raspberries and *R. occidentalis* (*R.* × *neglectus* Peck) (2007).

There is some disagreement about which red raspberry species was originally hybridized with *R. occidentalis* L. to produce the first described *R.* × *neglectus* Peck. (2007). There is also disagreement as to whether these interspecific hybrids should have a separate species name or whether they should follow the convention of naming hybrids such as *R. idaeus* × *R. occidentalis*. For brevity and the remainder of this literature review, European red raspberries will be referred to as *R. idaeus*, Eastern black raspberries will be referred to as *R. occidentalis*, and hybrids between these two species will be referred to as *R.* × *neglectus* as this appears to be the current scientific parlance.

Table 1.1: Cultivars used for trial, including their parentage. Color coordinated to species.

Name	Female Parent	Male Parent
Caroline	(Autumn Bliss × Glen Moy)	Heritage
Kiwigold	(Milton × Cuthburt)	Durham
Anne	Amity	Glen Garry
Heritage	(Milton × Cuthburt)	Durham
Mandarin	(*Rubus parvifolius* × Taylor)	Newburgh
Prelude	NY817(unp) [`Hilton` (unp)×`NY60` (unp) (`Durham` (unp)×`September`(unp)]×`	Hilton
Spinefree Willamette	Mutant of Willamette	
NY03-01p	Jewel OP	Double Delight or Heritage
Royalty	(Cumberland × Newburgh)	(Newburgh × Indian Summer)
8FBBR	Jewel	(Bristol × [([Black Knight × Heritage] × [Black Knight × Reveille]) × (SCRI 52B6 (*R. occ.)* × Autumn Bliss)]
Explorer	WS001	
Bristol	Watson Prolific	Honeysweet
Huron	Rachel	Dundee
Jewel	(Bristol × Dundee)	Dundee
Munger	Seedling selection of Schaffer	
Wes-O4	Tom's River NJ	[Bristol × (*R. sumatranus* x *R. hirsutus*)]
Earlisweet	Oregon-US 1725 (Haut × *R. leucodermis*)	open pollinated

*Red= *R. idaeus*, Purple= *R. x neglectus*, White= *R. occidentalis*, unp=unpatented, OP= open pollinated

1.2. Commercial Importance and Industry

Raspberries are the third most popular berry with consumers in the United States, after strawberries and blueberries, and the U.S. is the third largest producer in the world (Geisler 2012). While grown in many areas around the country, commercial production mainly occurs in California, Oregon and Washington. Red raspberries (*R. idaeus*) are the most commonly grown commercial raspberry. Black raspberries (*R. occidentalis*) are produced mainly on the West Coast. Oregon, the largest producer of black raspberries in the country, grew 1.8 million pounds of black raspberries in 2011 valued at $2.3 million, as they were used primarily for processing into frozen puree, juice concentrate or quick frozen whole berries. In comparison, California produced a total of 81 million pounds of red raspberries in 2011 valued at $200 million (Geisler 2012). Due to year-round demand for raspberries, domestic production is heavily augmented by Canadian, Mexican and Chilean imports which accounted for about 85% of the total raspberries consumed domestically (2002).

Local and pick-your-own raspberries offer a more diverse array of fruit—these farms grow red (*R. idaeus)* and black raspberries (*R. occidentalis*) as well as purple (*R.* × *neglectus*) and yellow raspberries (*R. idaeus*). Unlike commercially produced raspberries that are picked slightly under-ripe to increase shelf-life for shipping (Kruger et al. 2011), berries bought from these local farms tend to be picked eating-ripe.

1.3. Black Raspberries

First domesticated in the 1830's, *R. occidentalis* is native to the Eastern United States from southern Canada to the Carolinas (Michael et al. 2012). While demand for black raspberries is increasing due to new reports about their health benefits, production

has been decreasing due to disease pressure from Verticillium wilt (*Verticillium albo-atrum* Reinke & Berthold), anthracnose (*Elsinoë veneta* (Burkh.) Jenkins) and several plant viruses. The life span of field plantings has decreased from more than seven cropping seasons in 1949 to three or four currently (Dossett, Lee and Finn 2008). In the Northeast, orange rust (*Arthuriomyces peckianus* (Howe) Cummins & Y. Hirats) limits production of susceptible black raspberries (*R. occidentalis*) and purple raspberries (*R.* × *neglectus*). The fungus completes its lifecycle on exclusively *Rubus* sp. and there are no effective chemical controls. Removing infected wild *Rubus* sp. near plantings, roguing infected canes and leaves and controlling weed populations are the recommended controls (SLJ 2011).

Research to understand and improve black raspberries (*R. occidentalis*) has increased over the last decade, mainly focused on the genetic diversity of wild and cultivated plants and the heritability of traits with a goal towards improved black raspberry cultivars, with greater disease resistance. Weber looked at the genetic diversity of 14 cultivars using RAPD markers and concluded that genetic diversity was quite low. Five cultivars 'Cumberland,' 'Black Hawk,' 'John Robertson,' and two wild selections accounted for more than half of the variability. He concluded that *R. occidentalis* germplasm could be expanded by incorporating wild genotypes near the edge of their native habitat (Weber 2003). Expanding on that, the genetic diversity of both wild and cultivated *R. occidentalis* accessions were analyzed using simple sequence repeats (SSRs). On a neighbor-joining dendrogram, all of the cultivars and several wild accessions that may be mislabeled cultivars clustered very closely. Only Explorer, a primocane-fruiting cultivar, did not cluster with the others. This paper agrees that wild

germplasm should be explored as the source for introducing more genetic diversity to cultivated black raspberries (Dossett et al. 2012)

1.4. Fruiting Habit and Raspberry Morphology

Raspberries have a perennial crown and root system, which leads to their ability to fruit on floricanes, on primocanes or on both floricanes and primocanes depending on how they are pruned. Primocanes are new vegetative growth. Once those shoots overwinter, they are considered floricanes and will fruit. After the floricanes fruit, they are removed to allow the new growth of primocanes (Fennell 2012). There are numerous red varieties that can fruit late in the season on primocanes. Black (*R. occidentalis*) and purple (*R.* × *neglectus*) tend to fruit only on floricanes. Purple raspberries, due to their parentage, can be cropped twice in one season. Only recently have primocane-fruiting black raspberries become available. Explorer was the first cultivar, released by Peter Tallman. Since its release, issues with poor fertility have been discovered and the commercial release of this cultivar has been discarded (Tallman 2009).

The morphology of raspberries makes the fruit very delicate and difficult to manage after harvest. Raspberries are an aggregate fruit comprised of many drupelets (small drupes) attached to the torus above the receptacle. When the fruit is ripe, it detaches easily from the receptacle, and epidermal hairs help to stabilize the fruit (Reeve 1954). The exocarp is so thin that individual druplets cannot be separated without tearing them. Raspberries exhibit a double sigmoid growth curve, similar to that in peaches, with an initial increase in growth the ten days after bloom, then a 10-20 day leveling off of growth followed by a final swell (Robbins and Fellman 1993). Firmness has been studied extensively to determine its relationship to storageability. Cultivars vary greatly

by berry firmness. Cultivars with firmer berries at harvest will maintain their relative firmness in storage, and firm fruits are less susceptible to leakage after harvest (Robbins and Fellman 1993).

1.5. Post-Harvest Storage

Storage conditions that optimize shelf-life in red raspberries have been well documented; optimal storage conditions are -0.5 to 0°C at 90-95% relative humidity, with CO_2 levels at 45% (Robbins and Fellman 1993, Robbins, Sjulin and Patterson 1989, Kruger et al. 2011). These conditions can significantly slow ripening, respiration, desiccation, darkening, fungal spore germination and fungal growth without changing the flavor (Robbins and Fellman 1993).

The effects of different storage conditions on the chemical qualities of red raspberries have also been studied. Soluble solids, ascorbic acid, total phenols, total anthocyanin and antioxidant capacity were highest in slightly over-ripe fruit. Those chemicals also tended to increase during storage. Titratable acidity decreased as the fruit ripened and then remained relatively unchanged in storage (Kruger et al. 2011).

1.6. Ethylene Biology and its Role in Raspberry Ripening

Ethylene is the simplest plant hormone structurally, C_2H_4, and diffuses easily within the plant as a gas. While almost all plant tissues produce basal levels of ethylene, the increase in biosynthesis of ethylene in fruits is the physiological signal that induces ripening in climacteric species. Fruits are classified as being either climacteric or non-climacteric based on their ethylene evolution rates and their responses to exogenously-applied ethylene. Climacteric fruit show increased ethylene evolution and increased

respiration. When exogenous ethylene is applied, non-climacteric fruits show an increase in respiration without any hastening of the ripening process. Climacteric fruits will respond to ethylene application with accelerated ripening and increased respiration dependent on the concentration applied (Owino and Ezura 2008). There is not a consensus on whether raspberries are climacteric or non-climacteric fruit. Raspberry fruit ripening shows a typical climacteric response for ethylene evolution—as the fruit ripens, the rate of ethylene evolution increases (Robbins et al. 1989, Blanpied 1972). However, raspberries do not show the characteristic increase in respiration rate expected in climacteric fruits (Robbins and Fellman 1993). Perkins-Veazie looked at the respiration rate and ethylene evolution rate in red raspberries (*R. idaeus*) at different stages of ripening and had similar results: there is no spike in respiration as the fruit ripen. In fact, the respiration rate actually decreased as fruit ripened. Ethylene evolution increased as fruit turned from green to dark red. The conclusion from that work, however, was that *R. idaeus* is a non-climacteric fruit (Perkins-Veazie and Nonnecke 1992). Work elucidating the ethylene evolution rates of different parts of the raspberry fruit has shown that the receptacle produces approximately three times more ethylene than the drupelets (Iannetta et al. 1999). Since ethylene production is autocatalytic, the increased ethylene evolution in the receptacle may then induce ethylene production in the drupelets and probably hasten the abscission zone formation (Burdon and Sexton 1990). Raspberry fruits have a climacteric response to exogenous ethylene as well. Exogenous ethylene treatments resulted in the ripening and premature shedding of unripe fruit, but had no effect on the retention strength of ripe fruit, suggesting ripe fruit are producing enough ethylene to fully saturate the abscission responses (Burdon and Sexton 1990).

Recent work on the ethylene biology of raspberries has focused on the genetic control of ethylene biosynthesis and signaling. Biosynthesis of ethylene occurs via a metabolic pathway: methionine is converted to S-adenosyl methionine (SAM) by the enzyme methionine adenosyl transferase. SAM is then converted by ACC synthase (ACS) into 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is then oxidized by ACC oxidase (ACO) to produce ethylene. Once it is released within the plant, it is perceived by receptors and the process of fruit ripening and senescence begins (Paliyath and Murr 2008). ACC synthase and ACC oxidase have both been studied as means of controlling the ethylene biosynthesis pathway. In particular, aminoethoxyvinylglycine (AVG), which prevents ethylene synthesis, is a widely studied chemical and registered commercially as ReTain®. Commercially, this chemical is used mainly to prevent apples from abscising and softening.

Recently, the ACC synthase gene was cloned from *R. idaeus* cv. Royalty. ACC synthase expression was analyzed in the fruit through several ripening stages (Zheng and Hrazdina 2010a). The expected pattern in climacteric fruit is that the upregulation and increased expression of ACC synthase will begin during the early stages of ripening. In *R. idaeus*, it was seen that the expression of ACC synthase was not detected in the early stages of ripening and was also very low during late ripening. This led the researchers to conclude that raspberries exhibited non-climacteric ripening process (Zheng and Hrazdina 2010b).

To perceive ethylene, there are five ethylene receptors that have been found in the plants that have been studied so far, namely *Arabidopsis thaliana*: ETR1, ERS1, ETR2, EIN4, and ERS2. These receptors can be separated into several functional domains: a

transmembrane region responsible for ethylene binding, a GAF domain with unknown function, a histidine-aspartate 2 kinase domain and a receiver domain that may modulate the activity of a downstream factor (Owino and Ezura 2008). Work done in another drupe, peach (*Prunus persica*), showed that Pp-ETR1 was essential to fruit ripening but unrelated to ethylene evolution. On the other hand, Pp-ERS1 transcripts increased during ripening and were upregulated when exogenous ethylene was applied (Rasori et al. 2002). This has not yet been pursued in *Rubus sp*., but further analysis of the ethylene pathway could lead to more concrete conclusions about their ripening behavior.

1.7. *Botrytis cinerea* and other Postharvest Rots

Gray mold (*Botrytis cinerea* Pers.:F.) is one of the most common diseases of *Rubus* sp. worldwide. The teliomorph or sexual state, *Botryotinia fuckeliana,* is rarely found in nature and has never been observed on *Rubus* sp. (Staples and Mayer 1995). First described on strawberries in Louisiana, *Botrytis* was reported to cause up to 50% yield losses (Stevens 1914). In raspberry, primary inoculum comes mainly from the asexual conidia that arise from sclerotia on canes and the mycelia on mummified berries and dead leaves where it can live as a saprophyte, living on dead or decaying tissues. Dispersed mainly by wind and splashing water, the conidia land on the stigmatic surfaces and germinate. Hyphae grow intercellularly and the fungus becomes dormant inside immature fruit tissue. Once the fruit becomes ripe, the fungus resumes growth and the typical fruit rot symptoms appear. While this is the main method of infection, *B. cinerea* can also infect undamaged tissues using appresoria and the secretion of a cell wall degrading enzymes (Staples and Mayer 1995). Petal infection, stamen infection and

direct infection of the ripe fruit are not a major source of inoculum or yield loss. Only the latent infection of unripe fruit causes serious losses.

The fungus overwinters on weeds, dead canes and dead leaves and it performs best in cool, wet weather. Inoculum concentration in the fields increase rapidly about the time that the first fruit is ripening. It remains high through the rest of the season due to sporulation on exposed receptacles and overripe fruit that are left on the plant. Primocane-fruiting raspberries have less primary inoculum during harvest as there are no overwintering canes. Pre-harvest rots, however, can become a greater problem in primocane-fruiting cultivars due to the heavy rainfall during August and September (Bristow 1991).

Commercially, control of *B. cinerea* can be achieved by regular spraying of protectant fungicides starting at early bloom, such as Pristine (Boscalid + Pyraclostrobin), Switch (Cyprodinil + fludioxonil), and Captan (dicarboximide). The fungus is genetically variable and has become resistant to many fungicides. In conjunction with fungicides, field maintenance is the way to control gray mold. Removing dead leaves and canes, keeping weed pressure down, and pruning to promote an open canopy that allows for quick drying and ample air movement all help to reduce inoculum pressure (Jarvis 1962a). There are red raspberry cultivars with increased tolerance to this disease and the difference between cultivars has been well-documented. None of these are noteworthy in their resistance. (Aprea et al. 2010, Bristow 1991)

Recent work has focused on clarifying the cellular effects of *B. cinerea* infection and the plant's response to that infection. One example is the ability of *B. cinerea* to

deactivate plant defense chemicals. Saponins, so named because they form a soapy solution in water, are fungicidal compounds which are used by plants to defend against infection. Structurally, they have a steroid ring and three sugar units (Le Couteur and Burreson 2004). Many phytopathogenic fungi can detoxify these saponins by deglycosylation, or removal of the sugar units. *B. cinerea* was shown to have the ability to detoxify several specific saponins: α-tomatine, avenacin, avenacodises and digitonin. (Quidde, Buttner and Tudzynski 1999). Little work, however, has been done with raspberries to look at the saponins they produce.

It has also been shown that *B. cinerea* has an intricate relationship with the plant's physiological processes concerning ethylene. *B. cinerea* evolves large quantities of ethylene gas when in culture through the 2-keto-4-methylthiobutryic acid (KMBA) pathway. It was hypothesized that it used this ability to induce the plant to start senescing or ripening, making infection easier for the fungus. It has since been discovered that ethylene antagonizes *B. cinerea*; ethylene is released by the plant upon *B. cinerea* infection and it limits colonization. *Nicotinia benthamiana* leaves were treated with AVG in order to shut down the plant's ethylene production. That resulted in the disease progressing much faster. (Diaz, ten Have and van Kan 2002). In a separate experiment, it was discovered that *B. cinerea* induces ripening genes in susceptible tomato strains (Cantu et al. 2009). Many of these hypotheses have not yet been tested in *Rubus* sp.

For raspberry production, *B. cinerea* is the predominant post-harvest decay pathogen. There are several other fungi that can also cause post-harvest decays, particularly when fungicides have lessened competition from *B. cinerea*. *Rhizopus stolinifer* (Ehrenb.) Vuill. (domestically) and *Mucor piriformis* Leers (in Europe) are two

fungi that infect mature or damaged fruit directly. Called pin mold, these fungi cause affected berries to leak and deteriorate as they produce copious masses of white-grey, coarse hyphae that produce black zygosporangia (Davis 1991). Overwintering as saprophytes on dead and decaying material, inoculum in the field builds rapidly following the first infection of the season. Spread by rain splash and wind, infections often develop within the cavity left by the receptacle of harvested berries. There are no chemical controls for these fungi and field hygiene is the only recommended action. Cultivars differ in susceptibility, with firmer fruit and tissue-resistance seemingly being responsible for the differences (Davis 1991). Other fungal rots include *Alternaria* spp, *Cladosporium* spp., *Penicillium* spp, and *Colletotrichum gloeosporiodes*, but these are minor in comparison to *Botrytis* (Davis 1991).

1.8. Nutritional Analysis

Worldwide raspberry production could be increasing due to the numerous reports of their health benefits. Antioxidants, which help prevent free radical-induced oxidative stress that is associated with several toxic cellular processes, come mainly from total phenolics, which include anthocyanins and flavonoids (Tosun et al. 2009). Much work has been done to elucidate specific anthocyanins, phenolics and flavonoids produced by different *Rubus* species (Lee, Dossett and Finn 2012) as well as work to understand the physiological and environmental conditions that affect antioxidant levels with the goal of using this information to improve variety development (Dossett, Lee and Finn 2010). It is generally thought that the darker the fruit, the greater the concentration of antioxidants. This has led to a recent, renewed research interest in *R. occidentalis* and its use in breeding programs.

1.9. Research Goals

The goal of this research was to look at the effect of storage temperature on disease incidence of different raspberry species, berry colors and genotypes. The temperatures chosen represent that traditionally used to screen raspberry selections in a breeding program (25°C) and that available to local, direct marketers that would typically be producing species of raspberry other than *R. idaeus* (5°C). It has been shown that *R. occidentalis* has a greater capacity to resist post-harvest decay. The role that ethylene plays in the resistance to post-harvest decay has not been well studied in raspberries. Using gas chromatography, this study aims to clarify that role. At the end of this study, the goal is to be able to make recommendations to raspberry breeders in an effort to create varieties that will have better post-harvest resistance to decay.

Chapter 2: Post-Harvest Biology and Technology Journal Manuscript

2.1 Introduction

Raspberries (*Rubus* sp.) are a growing specialty crop for both the wholesale industry and smaller, local markets and U-pick farms due to recent reports about the health benefits and the movement to eat locally grown food. Raspberries are the third most popular berry in the United States (Geisler 2012). Post-harvest susceptibility to gray mold (*Botrytis cinerea*) drastically reduces the shelf-life of this delicate fruit. While the wholesale commercial industry has optimized a pre- and post- harvest fungicide application program, harvesting the berries under-ripe, controlled atmosphere storage regime and cold chain management system to achieve maximum shelf-life (Robbins and Fellman 1993), smaller producers are far more vulnerable to losing yields due to gray mold. Local producers are less likely to pursue such aggressive fungicide regimes to control gray mold pre-harvest or to have the cold storage equipment necessary to increase shelf-life. Shelf-life of local sales is further compromised by harvesting berries when they are eating-ripe.

Three species of raspberries are commonly grown at local markets: the European red raspberry (*R. idaeus* L.) that is usually red but is also available in yellow, the eastern black raspberry (*R. occidentalis* L.) and an interspecific hybrid between the two (*R.* x *neglectus* Peck.) that yields purple berries. Little research has been done to compare the storeageability of these different types of raspberries, particularly when picked ripe for local markets. Black raspberries are primarily grown commercially in Oregon where they face many disease pressures that reduce annual yields and shorten the life of plots (Dossett et al. 2008). That problem is exacerbated by the recent discovery that the genetic

diversity of commercially cultivated black raspberries is incredibly low, with 95% of the variation accounted for by five varieties (Weber 2003).

Physiological characteristics of raspberries, such as fruit chemistry, firmness and color, and ethylene biology have been studied in their relationship to post-harvest quality and disease resistance. There are few straightforward conclusions about berry firmness, except that cultivars with firmer berries at harvest will maintain their relative firmness in storage. Firm fruits are also less susceptible to leakage after harvest (Robbins and Fellman 1993). As for fruit chemistry, there has recently been extensive work showing the concentration of various compounds (anthocyanins, phenolics, titratable acids, soluble solids, antioxidant capacity) at different ripeness stages and the changes in concentrations during storage (Kruger et al. 2011). Red raspberries show an ethylene evolution peak analogous to a climacteric fruit, but there is no concomitant rise in the fruit respiration rate, which corresponds to non-climacteric fruit (Robbins and Fellman 1993, Perkins-Veazie and Nonnecke 1992, Iannetta et al. 1999). Recent molecular work also supports that red raspberries are a non-climacteric fruit. The expression level of the regulatory enzyme ACC synthase was not detected in early ripening stages and only at very low levels in later ripening stages, which is commonly observed in non-climacteric fruit (Zheng and Hrazdina 2010b).

The biology of *B. cinerea* on *Rubus* sp. has been well studied (Jarvis 1962b). Primary inoculum comes from conidia that arise from sclerotia that overwinter on dead or decaying material such as leftover floricanes. In this tissue, the pathogen lives as a saprophyte. Conidial spores are spread by wind and splashing water and they germinate in the stigmatic fluid upon landing on the floral surface. The hyphae grow intercellularly

within unripe fruit and remain dormant. Once the fruit begins to ripen, the fungus resumes its growth and the typical rot symptoms appear. Inoculum concentration increases as the first crop ripens and remains high through the rest of the season. Control is achieved through the use of fungicides and field maintenance. Removing dead leaves and canes, keeping weed pressure down, and pruning to promote an open canopy that allows for quick drying and ample air movement all help to reduce inoculum pressure (Jarvis 1962a).

Much is known about red raspberry production, *B. cinerea* life cycle and control and the presence of ethylene in red raspberries. As production of alternate raspberry species increases for direct markets and commercially for the health benefits, more information about post-harvest resistance and physiological characteristics are needed. The goal of this study was to compare the post-harvest quality of different species, berry colors and genotypes of raspberries that were grown without fungicides or insecticides in areas with high summer rainfall under storage conditions that would be available to small, direct market farms.

2.2 Materials and Methods

2.2.1. Field Layout.

Thirty cultivars of raspberries were randomized within in two replicated blocks at USDA-ARS Beltsville, MD in August of 2007 (Figure 1.1). Each plot of raspberries contained five plants, 1 meter apart, and 3 meters in-row spacing. The thirty cultivars planted consisted of both named varieties and breeding selections. Released varieties were purchased from Indiana Berry (Plymouth, IN), Nourse Farms (Whatley, MA), and

Hartman's (Lacota, MI) over the course of 2006 and 2007. Breeding selections were obtained from Cornell University (Courtney Weber, NY), The University of Maryland (Harry Swartz, MD), and North Carolina State University (Gina Fernandez, NC.) Fertilization was applied through a drip irrigation system. Weed control was achieved using Surflan (Oryzalin) as a pre-emergent herbicide, Paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride) at a post-emergent at the base of the plants in the spring and hand weeding. Nitrogen was applied twice, one in the spring and once in July between crops, each time at 11kg N per hectare (60lbs per acre). While plots were irrigated and fertigated as needed, they were not treated for disease or insects at any time during this experiment. Plots were trellised in 2010 using a double-t trellis individually to accommodate the different growth habits. All plots were pruned for both floricane and primocane fruit production; pruning was done once in mid-summer to remove spent floricanes and once in early spring to train the primocanes. Due to differences in vigor and precocity, only 17 genotypes that previously had high yields were selected for use in the postharvest experiments (Table 2.1).

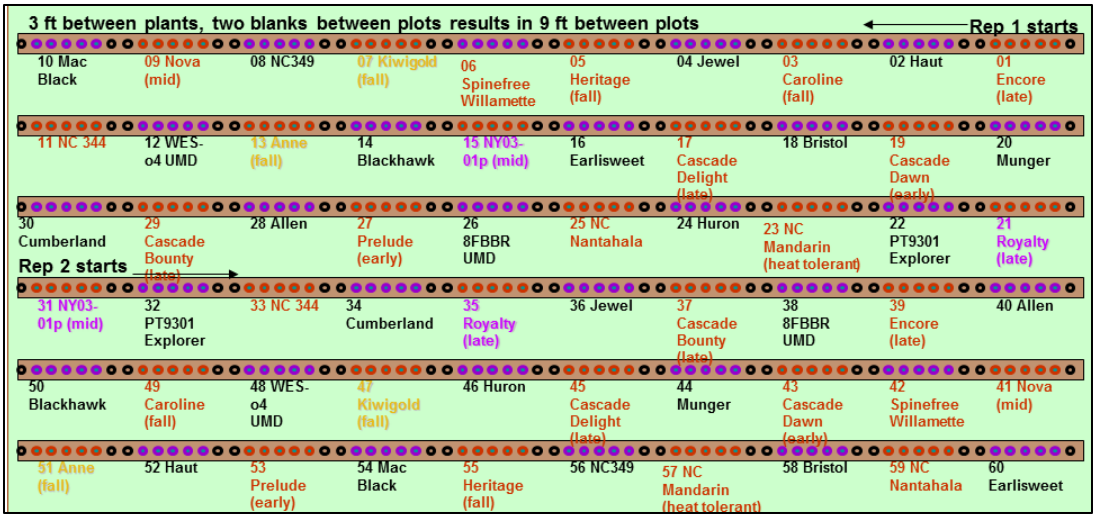

Figure 2.1. Field Layout showing Genotypes, Berry color (indicated by font color) and blocks, which are the same as the replications.

Table 2.1. Species, Berry Color and Fruiting Season for all Genotypes Studied

Genotype	Species	Berry Color	Fruiting Season
Anne	*R. idaeus* L.	Yellow	Flori, Primo
Caroline	*R. idaeus* L.	Red	Flori, Primo
Heritage	*R. idaeus* L.	Red	Flori, Primo
Kiwigold	*R. idaeus* L.	Yellow	Flori, Primo
Mandarin	*R. idaeus* L.	Red	Flori, Primo
Prelude	*R. idaeus* L.	Red	Flori, Primo
Spinefree Willamette	*R. idaeus* L.	Red	Flori
NY03-01	*R. neglectus* Peck.	Purple	Flori
Royalty	*R. neglectus* Peck.	Purple	Flori
8FFBBR	*R. neglectus* Peck.	Purple	Flori, Primo
Bristol	*R. occidentalis* L.	Black	Flori
Earlisweet	*R. occidentalis* L.	Black	Flori
Explorer	*R. occidentalis* L.	Black	Flori, Primo
Huron	*R. occidentalis* L.	Black	Flori
Jewel	*R. occidentalis* L.	Black	Flori
Munger	*R. occidentalis* L.	Black	Flori
Wes-04	*R. occidentalis* L.	Black	Flori

*Flori= floricane-fruiting, Primo=primocane-fruiting

2.2.2. Sampling techniques

Floricane fruiting began in early June and continued for about a month.

Primocanes began fruiting in early July and ended about the first week of September.

Raspberries were harvested twice a week throughout the season in both years. Extra care was taken to reduce the introduction of inoculum between plots by wearing gloves and surface sterilization of gloves with an ethyl alcohol sanitizer between plots. Raspberries were harvested when they were fully ripe, meaning they detached from their receptacles easily. Berries that were overripe or that were showing signs of fungal infection were not picked. These berries were harvested into 12-well Corning® Costar® plates lined with filter paper. Full plates were closed, field cooled briefly in an insulated cooler and moved to their treatment area within about an hour of harvest.

2.2.3. Shelf-life Tests

Two plates for each plot were placed in a walk-in cooler at 5°C and in an incubator at 25°C. These two temperatures were chosen to mimic the cold-chain management conditions of direct-market producers (5°C) and conditions that fruit breeders sometime use to test potential selections (25°C). Plates were sealed with ParaFilm® 3M strips. Disease incidence—number of berries bleeding and number of berries showing signs of fungal infection— was recorded every other day during the two weeks after harvest. A berry was considered to be bleeding if, when looking at the bottom of the Costar plate, any spotting was seen on the filter paper. A berry was considered to be show signs of fungal infection if mycelia could be seen without the aid of a microscope. Desiccation and insect damage, while seen and noted, were not scored.

To analyze these data, straight line equations between the two measurements that most closely bracketed 25% decay or leakage were used for each Costar® plate. The threshold of 25% was chosen to reflect a number that would be easily visible to a consumer, beyond the grower's ability to repack to remove contamination and,

experimentally, to protect plates from being immediately discarded that contained a single infected berry accidentally picked for trial that might not reflect the true storageability of the cultivar or selection.

2.2.4. Koch's postulates and Genetic ID of *Botrytis cinerea*

Botrytis cinerea was isolated from symptomatic raspberries from experimental plots (located and maintained at BARC (Beltsville Agricultural Research Center, Beltsville, MD) and maintained on PDA (potato dextrose agar). The isolate was identified as *B. cinerea* both by cultural morphology and intertranscribed ribosomal RNA spacer (ITS) gene sequence. Conidia were harvested by washing plates containing the sporulating fungus with 2-3mL of sterilized distilled water containing Tween 20 surfactant (1µL Tween 20/1mL water.) The conidial suspension was quantified using a hemacytometer and adjusted to 10^4 concentration with sterile distilled water. Raspberries were surface sterilized with 70% ethanol and allowed to air dry in a flow hood before being inoculated. Sterilized raspberries were inoculated with 10µL of a 10^4/ml conidial spore suspension of *B. cinerea* (Celik et al. 2009). The inoculated raspberries were placed at 25°C and once mycelial growth was visualized, the fungus was cultured from the infected fruit on to potato dextrose agar plates as well as 50 ml potato dextrose broth (PDB) liquid cultures that were used to extract genomic DNA from the mycelia. Fungal identification of the grey mold isolate was carried out at the genus and species level by visually identifying the cultural characteristics (i.e. condia, cultural morphology etc.) and by sequencing the ITS1 (intertranscribed ribosomal RNA spacer) locus.

The complete nucleotide sequence for the ITS1 locus was obtained from *B. cinerea* GenBank accession GU395993.1. The following, gene specific primer set was

used to perform conventional PCR: Forward- 5’ GAT GCC CGA AAG GGT AGA 3’; Reverse- 5’ ATA TAG TAC TCA GAC GAC ATT 3’. Mycelial mats were grown in PDB liquid shake cultures at 150rpm in Innova 4230 refrigerated incubator shaker incubators (New Brunswick Sci, Edison, NJ) at 25°C. Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) following the manufacturer's protocol. The quantity and quality of the genomic DNA was assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). The PCR cycling parameters were as follows: initial denaturation for 3 min at 95°C; 30 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were loaded on a 1% TBE agarose gel with containing ethidium bromide and electrophoresed at 100 V for 50 min according to standard procedures as outlined in Sambrook and Russell, 2001. Bands on the gel were visualized using an Alpha imager mini UV light box (Model M-26E; Alpha Innotech Corporation). The PCR products were purified using QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA). The purified products were sequenced directly by Macrogen Inc. (Rockville, MD) using the Sanger method and the gene specific primers that were used to generate the PCR amplicon.

2.2.5. Firmness and Color

Berries harvested for fruit quality measurements were analyzed once on the day of harvest and then again six days after harvest. Plates were weighed at harvest to obtain the mean berry weight. Color was measured at two points on six of the twelve berries using a portable *L*a*b* colorimeter Chroma-Meter CR 400 (Minolta, Germany). The colorimeter was calibrated to a white reflective plate (L*=97.93, a*= -0.34 *b**=2.27, standard

illumination C). L* is a measure of lightness from 0=black and 100-white. The a* value represents red-green color with positive values being red and negative values being green. The b* value represents yellow-blue color with positive values being yellow and negative values being blue. Firmness measurements were taken on those same six berries using the TA-25 tip on a TA XT-plus Texture Analyzer (Stable Micro Systems [SMS], Godalming, UK) by placing the berry calyx-side down. This test is destructive, so berries were discarded after analysis. The remaining six berries were stored at 5°C until day six.

2.2.6. Gas Chromatography

Raspberries collected for gas chromatographic analysis were harvested using sterile techniques, weighed and then sterilized with a 70% ethanol solution. The fruit were then placed in sterilized 8 ounce canning jars (236.59 mL) and labeled by plot number. The jars were cooled to 5°C and the first measurements were taken approximately six hours after harvest. Carbon dioxide and ethylene evolution rates were measured by sealing the jars for 1 hour and then sampling 25mL of the headspace. Carbon dioxide was determined using a GC (model GC-3BT; Shimadzu, Kyoto, Japan) fitted with Porapak Q and molecular sieve5A columns (2m x 3mm) and a thermal conductivity detector. Ethylene was measured with a GC (model AGC-211; Carle, Tulsa, OK) fitted with an alumina column (2m x 3mm) and a flame ionization detector. Measurements were taken every 24 hours until the point where further data was unusable due to fungal decay.

2.2.7. ORAC analysis

pH. Raspberry juice was extracted from 400g samples manually using cheesecloth. The pH of raspberry juice was determined using a Denver pH meter (Bohemia, NY). Using a

digital refractometer, soluble solids of the fruit was determined at 20°C (PR-101, Spectrum Technologies, Plainfield, IL). Titratable acidity was measured by first diluting each 5-ml aliquot of raspberry juice to 10ml with distilled water and then adjusting the pH to 8.2 using 0.1N NaOH. Acidity was expressed as percent of citric acid equivalent.

Total Phenolic Content and Anthocyanin Assay. Using a Polytron homogenizer, 5g of raspberries were extracted twice with a 15mL solution of 80% acetone with 0.2% formic acid (Brinkman Instruments, Westbury, NY). Those samples were then centrifuged at 4°C for 20 minutes. The extracts were combined and then transferred to vials and stored at -80°C to be used later for total anthocyanin and total phenolic measurements.

Total anthocyanin content in raspberry extract was measured using the pH differential method (Cheng and Breen, 1991). Absorbance was measured with a spectrophotometer (UV-160; Shimadzu Scientific Instruments, Columbia, MD) at 510 nm and 700 nm in pH buffers at 1.0 and 4.5, using A =[(A510 – A700) pH 1.0 (A510 – A700) pH 4.5] with a molar extinction coefficient of cyanidin 3-glucoside (26, 900 L/cm-mol) for raspberry fruit extracts. Results were expressed as milligrams of cyanidin 3-glucoside equivalent, in the raspberry fruit extract, per 100 g fresh weight.

Total phenolic content in the fruit extract was measured with Folin-Ciocalteu reagent. Solid-phase extraction procedures were used to remove water soluble compounds from the extracts initially, as the Folin-Ciocalteu reagent is affected by them. Five mL from the above extracts were concentrated to 1 mL using a Buchler Evapomix (Fort Lee, NJ) in a water bath at 30 ºC. The concentrated samples were dissolved in 4 mL of acidified water (3% formic acid) then passed through a Sep-Pak C18 cartridge

(Waters), which was previously activated with methanol followed by water and 3% aqueous formic acid. The interfering substances, sugars, ascorbic acid, organic acids, and non-phenolic organic substances, which react with Folin-Ciocalteu passed through the Sep-Pak C18 column. Anthocyanins and other phenolics were retained by the column and then recovered with 5.0 mL of acidified methanol containing 3% formic acid. Total phenolics were then determined with Folin-Ciocalteu reagent by the method of Slinkard and Singleton (1997) using gallic acid as the standard. Results were expressed as milligrams gallic acid equivalent (GAE), in the raspberry extract, per 100 gram fresh weight.

Antioxidant Capacity for Lipophilic and Hydrophilic [Oxygen Radical Absorbance Capacity (ROOFL)] Assay. Five grams of raspberry fruit were extracted twice with 10 mL of hexane and then centrifuged at 7,500 g for 20 min at 4 °C. The two hexane extracts were removed and then combined. Residual hexane was evaporated from the original tube, and the residue was extracted with 2 x10 mL of acetone/water/acetic acid, (70:29.5:0.5, v/v/v). After adding solvent, the tube was vortexed for 30 s and then sonicated for 5 min at 37 °C. The tube was left at room temperature for 10 min and occasionally shaken. Then the tube was centrifuged at 7, 500 g for 15 min. The supernatant was removed and the total volume was bought up to 25 mL with acetone/water/acetic acid (70:29.5:0.5, v/v/v). Any further dilution of the hydrophilic fraction was made with phosphate buffer for hydrophilic antioxidant assay.

The combined hexane fractions from above were dried using a Buchler Evapomix (Fort Lee, NJ) in a 30 °C water bath. The dried hexane extract was dissolved in 250 μL of acetone and then diluted with 750 μL of a 7% RMCD solution (50% acetone/50%

water, v/v) for the lipophilic antioxidant assay. Any further dilution was with the same solution. The 7% RMCD solution was used as a blank and to dissolve the Trolox standards for the lipophilic antioxidant assay.

The ORACFL assay for hydrophilic and lipophilic antioxidant assay was carried out according to Huang et al. (2002) using a high-throughput instrument platform consisting of a robotic eight-channel liquid handling system. A microplate fluorescence reader (FL800; Bio-Tek Instruments, Winooski, VT) was used with fluorescence filters for an excitation wavelength of 485 ± 20 nm and an emission wavelength of 530 ± 25 nm. The ORAC values were determined by calculating the net area under the curve (AUC) of the standards and samples (Huang et al., 2002). The standard curve was obtained by plotting Trolox concentrations against the average net AUC of the two measurements for each concentration. Final ORAC values were calculated using a regression equation between Trolox concentration and the net AUC, and were expressed as micromole Trolox equivalents per gram fresh weight (Huang et al., 2002; Prior et al., 2003).

2.2.8. Statistical Analysis

Statistical analysis was performed using SAS Software, version 9.2 (SAS Institute Inc., Cary, NC, USA). Disease incidence was analyzed by analysis of variance (ANOVA) to determine the effects of species, berry color and genotype. Mean separations were carried out using the Tukey method when the treatment effect was statistically significant ($P< 0.05$). Stepwise regression was used to analyze the weather data, separated by fruiting season, using the Regression procedure. Multivariate analysis was done with exploratory factor analysis using the Factor procedure (Proc Factor). This was done in an

attempt to find possible underlying dimensions that could explain the interrelationships among response variables.

2.3. Results

Originally, the goal of this experiment was to determine the effect of storage temperature on the ability to detect difference in disease incidence among species, berry colors and genotypes. The interaction between storage temperature and species was significant for both number of days to 25% bleed ($P<0.0001$) and days to 25% rot ($P<0.0001$). A separate analysis shows that the interaction between storage temperature and berry color is also significant for both measures of disease incidence ($P<0.0001$). Further analysis shows that the same is true also for genotypes and temperature ($P<0.0001$). Because of these significant interactions, the analysis of species, berry colors and genotypes were separated by temperature.

2.3.1. Warm Storage

2.3.1.1. Interaction

There was a significant interaction between harvest season and species ($P=0.0433$), and harvest season and berry colors ($P=0.0398$) for incidence of rot, therefore the analysis were separated by season. Interaction between harvest season and genotype was not significant for incidence of rot but it was for incidence of bleed ($P<0.0001$). Because the same genotypes were not harvested in both seasons, results by genotype were also split by harvest season.

2.3.1.2. Floricane-Harvests

When incidence of rot is examined by species for floricane-harvests, *R. occidentalis* is able to resist rot the longest, and then *R.* × *neglectus* (Table 2.2). *R. idaeus* is least able to resist rot and stores for a significantly shorter period. If the same data are analyzed by berry color, there are no significant differences and the order in which the colors resist rot remains the same, with yellow berries rotting more quickly than red berries. Analyzed by genotype, Munger and Bristol, both *R. occidentalis* genotypes, are the two that take longest to reach the disease threshold (Table 2.3).

When bleed incidence is examined based on species for floricane-harvests, *R. idaeus* is the last species to show visible bleeding (Table 2.2). It resists post-harvest leakage significantly longer than either *R.* × *neglectus* or *R. occidentalis.* Examined by berry color, only yellow resists leak significantly longer than either of the other three fruit colors. It was observed, as in the cold-storage data, that the average days to 25% bleed for *R. idaeus* was not indicative of either color's true resistance. Examined by genotype, only Kiwigold is significantly different from the other genotypes. It takes the longest to reach 25% bleed at 9.57 days (Table 2.3). Interestingly, the other yellow genotype Anne does not show the same ability to resist bleed in warm storage. The overall trend seems to be that the red raspberry (*R. idaeus*) genotypes took longer to reach the bleed threshold than either purple (*R.* × *neglectus*) or black raspberry (*R. occidentalis*) genotypes.

2.3.1.3. Primocane-Harvests

When analyzed by species, incidence of rot is not significant. *R.* × *neglectus* lasts longest at 2.11 days, followed by *R. idaeus* and lastly *R. occidentalis* (Table 2.4). When analyzed by berry color, incidence of rot is significant (P=0.0291). Purple raspberries

resist rot the longest at 2.10 days, followed by red raspberries, then black raspberries and lastly yellow raspberries (Table 2.4). Yellow raspberries are significantly different only from red raspberries. There were no significant differences when genotype was analyzed (Table 2.5). Caroline, a red raspberry, followed by 8FBBR, a purple raspberry, both took slightly more than two days to reach the rot threshold. All other genotypes lasted less than two days.

The incidence of bleed by species in primocane-harvests shows that there are no significant differences. *R. idaeus* resists leakage the longest at 2.3 days (Table 2.4). Analyzed by berry color, the data shows that yellow berries had greatest storageability, which was significantly longer than red berries. Red raspberries are not statistically different from either purple or black colored raspberries. Examined by genotype, Kiwigold is again the genotype to resist bleed the longest at 3.56 days (Table 2.5). It is significantly different from only Caroline and Heritage.

Table 2.2: LS Means of disease incidence of field grown, floricane-harvested raspberry species and berry colors stored at 25°C for 2010 and 2011. Means followed by the same letter are not significant different (α=0.05).

Species	Days to 25% Rot	Berry Color	Days to 25% Rot	Days to 25% Bleed	Berry Color	Days to 25% Bleed
R. occidentalis	4.59 a	Black	4.59 a	2.14 b	Black	2.12 b
R. × *neglectus*	3.45 a	Purple	3.45 a	2.44 b	Purple	2.44 b
		Red	2.68 a	4.75 a	Red	3.41 b
R. idaeus	2.65 b	Yellow	2.61 a		Yellow	7.23 a

Table 2.3: LS Means of disease incidence of field grown, floricane-harvested raspberry genotypes stored at 25°C for 2010 and 2011. Means followed by the same letter are not significant different (α=0.05).

Genotype	Species	Days to 25% Rot	Days to 25% Bleed
Munger	*R. occidentalis*	7.14 a	2.42 b
Bristol	*R. occidentalis*	6.99 a	2.94 b
Royalty	*R.* × *neglectus*	4.99 ab	3.00 b
Prelude	*R. idaeus*	4.11 ab	2.98 b
Caroline	*R. idaeus*	3.86 ab	3.32 b
Huron	*R. occidentalis*	3.44 ab	1.50 b
Earlisweet	*R. occidentalis*	3.23 ab	3.17 b
Explorer	*R. occidentalis*	3.22 ab	2.63 b
Wes-04	*R. occidentalis*	3.22 ab	2.42 b
Anne	*R. idaeus* (yellow)	2.98 ab	2.89 b
Jewel	*R. occidentalis*	2.95 b	1.32 b
NY03-01	*R.* × *neglectus*	2.94 b	1.33 b
Spinefree Willamettee	*R. idaeus*	2.91 b	3.03 b
8FBBR	*R.* × *neglectus*	2.39 b	2.46 b
Kiwigold	*R. idaeus* (yellow)	2.36 b	9.57 a
Mandarin	*R. idaeus*	2.36 b	4.75 b
Heritage	*R. idaeus*	1.81 b	2.81 b

Table 2.4: LS Means of disease incidence of field grown, primocane-harvested raspberry species and berry colors stored at 25°C in 2010 and 2011. Means followed by the same letter are not significant different (α=0.05).

Species	Days to 25% Rot	Berry Color	Days to 25% Rot	Days to 25% Bleed	Berry Color	Days to 25% Bleed
R. occidentalis	1.52 a	Black	1.52 ab	1.86 a	Black	1.85 ab
R.× neglectus	2.11 a	Purple	2.10 ab	2.05 a	Purple	2.05 ab
		Red	2.06 a	2.30 a	Red	1.59 b
R. idaeus	1.71 a	Yellow	1.26 b		Yellow	3.21 a

Table 2.5: LS Means of disease incidence of field grown, primocane-harvested raspberry genotypes stored at 25°C in 2010 and 2011. Means followed by the same letter are not significant different (α=0.05).

Genotype	Species	Days to 25% Rot	Days to 25% Bleed
Caroline	*R. idaeus*	2.57 a	1.58 b
8FBBR	*R. × neglectus*	2.11 a	2.05 ab
Heritage	*R. idaeus*	1.79 a	1.52 b
Explorer	*R. occidentalis*	1.51 a	1.86 ab
Anne	*R. idaeus* (yellow)	1.45 a	2.58 ab
Mandarin	*R. idaeus*	1.34 a	3.35 ab
Prelude	*R. idaeus*	1.29 a	1.95 ab
Kiwigold	*R. idaeus* (yellow)	1.18 a	3.56 a

2.3.2. Cold Storage

2.3.2.1. Interactions

The interaction between floricane-fruiting harvests and primocane-fruiting harvests was significant (P=0.0268) for species. This is due to the fact that different genotypes were being evaluated in each season (Table 2.1) –black and purple raspberries do not tend fruit on primocanes. When only the cultivars that harvest in both seasons are compared, season effect is not significant for rot (P=0.2785) or for bleed (P=0.5199). The trend for both seasons when comparing rot incidence is that *R. occidentalis* takes the longest time to reach the threshold, followed by *R.* × *neglectus*, and lastly *R. idaeus* *(*Figure 2.2). One exception is that in the primocane-fruiting season, *R.* × *neglectus* takes longest to reach the 25% threshold. This change was most likely due to primocane-fruiting purple raspberries (*R.* × *neglectus*) being represented by only 8FBBR and primocane-fruiting black raspberries (*R. occidentalis*) being represented by only Explorer. The raspberries tended to rot more quickly in the primocane-fruiting harvests. The relationship between species for bleed incidence remains the same for both harvest seasons: *R. idaeus* takes the longest to reach 25% bleed, then *R. occidentalis* and then *R.* × *neglectus* (Figure 2.2). In this case, there seems to be no difference between season in the time it took the raspberries to reach 25% bleed.

The same data set analyzed by berry color shows a similar trend for rot incidence. Black raspberries took longest to reach the 25% threshold followed by purple raspberries, then red and lastly yellow raspberries (Figure 2.3). The only exception is that purple raspberries took longest to reach the threshold when fruiting on primocanes. For bleed incidence, yellow berries take the longest time to reach the 25% threshold for both

fruiting seasons. Purple berries and black berries are almost indistinguishable for either season. Red raspberries take longer to reach the bleed threshold when fruiting on floricanes, but perform similarly to the black and purple raspberries when fruiting on primocanes (Figure 2.3). Despite the above noted problem with the primocane-fruiting black raspberry, there is still a notable season effect.

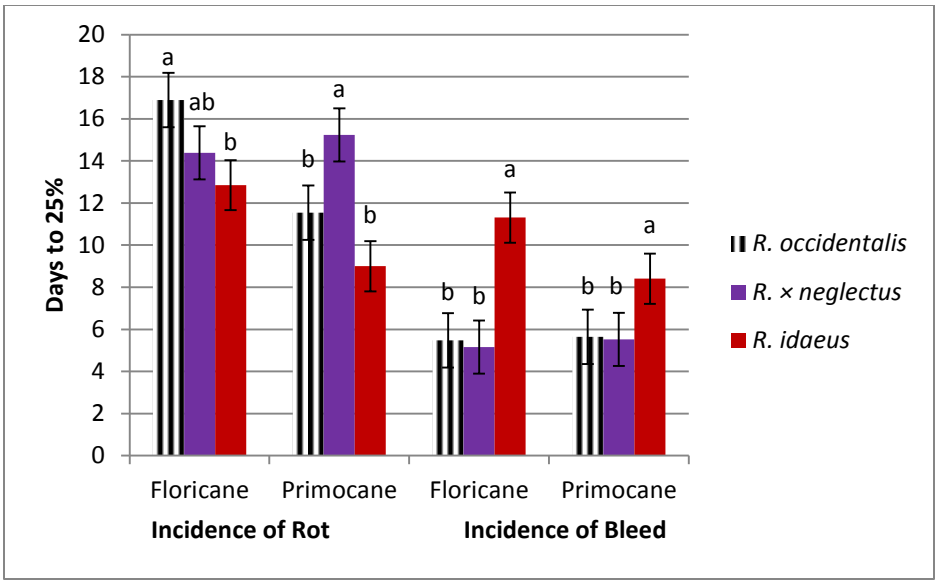

Figure 2.2: LS Means of Days to 25% Rot (Left panel) and Days to 25% Bleed (Right panel) of field grown raspberry species stored at 5°C in 2010 and 2011. For each set of bars, means followed by the same letter are not significant different (α=0.05). Error bars are one standard error.

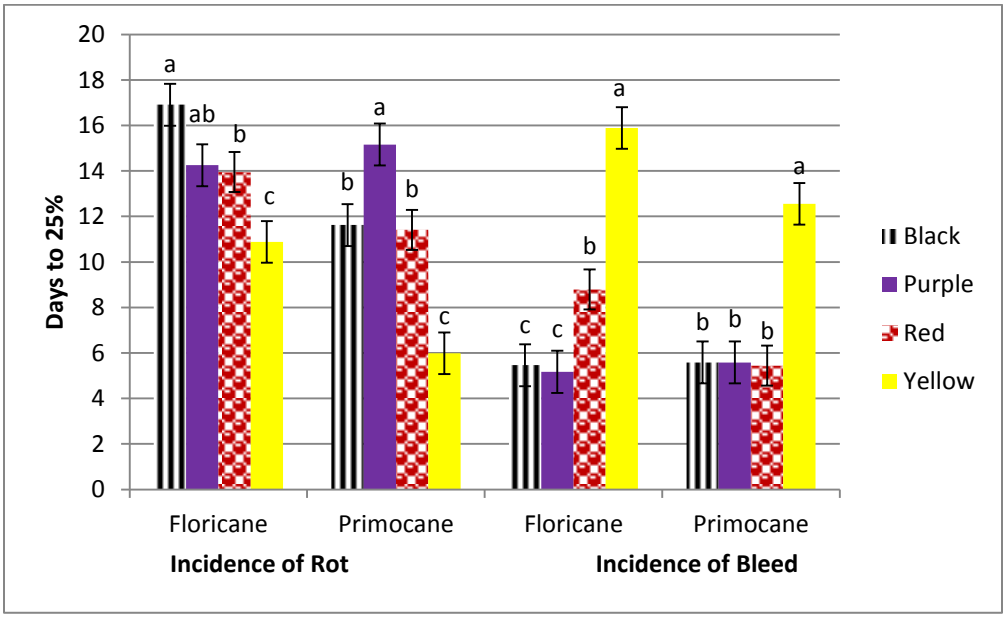

Figure 2.3: LS Means of Days to 25% Rot (Left panel) and Days to 25% Bleed (Right panel) of field grown raspberries by berry color stored at 5°C in 2010 and 2011. For each set of bars, means followed by the same letter are not significant different (α=0.05). Error bars are one standard error.

2.3.2.2. Floricane-Fruiting Harvests

When species is analyzed, incidence of rot for berries stored at 5°C was significant (P<0.0001). Only *R. occidentalis* and *R. idaeus* were significantly different (Figure 2.2). When berry color is analyzed, rot incidence is also significant (P<0.0001). Mean separation tests show that black raspberries were significantly different from red and yellow raspberries, but not from purple raspberries (Figure 2.3). Red raspberries were significantly different from yellow raspberries. Berry color highlighted the large differences between red and yellow within *R. idaeus*, showing that the mean value for *R. idaeus* was not indicative of either color fruit's behavior. Genotype analysis revealed that Spinefree Williamette (*R. idaeus*) was the genotype that stored the longest after Explorer (Table 2.6). 8FBBR, a purple raspberry, performed most similarly to the black raspberries in its ability to resist post-harvest decay. Royalty and NY03-01, the other two *R.* × *neglectus* genotype performed more similarly to the red genotypes.

Species was significant (P<0.001) when comparing the average number of days to 25% bleed (Figure 2.2). *R. idaeus* was significantly different from the other two species. Berry color was also significant for bleed incidence (P<0.001). Berry color yielded the opposite results from the time to 25% rot (Figure 2.3); yellow raspberries last the longest and are significantly different from all other berry colors. When genotype is analyzed, the two yellow genotypes, Anne and Kiwigold, took the greatest number of days to reach 25% bleed and were significantly different from most of the other genotypes (Table 2.6). Several genotypes did not make it three days in storage before reaching the threshold.

Table 2.6: LS Means of disease incidence of field grown, floricane-harvested raspberry genotypes stored at 5°C in 2010 and 2011. Means followed by the same letter are not significant different (α=0.05).

Genotype	Species	Days to 25% Rot	Days to 25% Bleed
Explorer	*R. occidentalis*	18.43 a	6.28 cd
Spinefree Willamette	*R. idaeus*	17.88 a	7.20 cd
Bristol	*R. occidentalis*	17.85 a	7.90 cd
8FBBR	*R.* × *neglectus*	17.81 a	7.36 cd
Jewel	*R. occidentalis*	17.49 ab	2.64 d
Huron	*R. occidentalis*	16.98 ab	2.41 d
Earlisweet	*R. occidentalis*	16.33 abc	7.11 cd
Munger	*R. occidentalis*	16.24 abc	8.65 cd
Caroline	*R. idaeus*	14.51 abc	7.32 cd
Mandarin	*R. idaeus*	14.18 abc	10.33 bc
Heritage	*R. idaeus*	13.12 abc	9.72 bc
Royalty	*R.* × *neglectus*	12.85 abc	3.92 d
Kiwigold	*R. idaeus* (yellow)	11.44 abc	16.03 a
Wes-04	*R. occidentalis*	11.1 abc	4.10 cd
NY03-01	*R.* × *neglectus*	9.58 abc	2.39 d
Anne	*R. idaeus* (yellow)	9.57 c	15.47 ab
Prelude	*R. idaeus*	8.48 b	4.44 cd

2.3.2.3. Primocane-Fruiting Harvests

Species and berry color both have a significant effect on the average number of days to 25% rot for primocane-fruiting harvests (P>0.0001). When separated by species, *R. neglectus* was significantly different from *R. occidentalis* and *R. idaeus,* but *R. idaeus* and *R. occidentalis* were not significantly different from each other. When separated by berry color, the same relationships were seen and yellow raspberries are significantly different from all other three colors of fruit (Figure 2.2). It is important to note that black

raspberries were represented exclusively by Explorer, which at the time of this planting, was the only primocane-fruiting black raspberry available.

Species (P=0.0033) and berry color (P>0.0001) had a significant effect on the average number of days to 25% bleed. Separated by species, *R. idaeus* took the longest to reach that threshold and was significantly different from the other two species. *R. x neglectus* and *R. occidentalis* were not significantly different from one another. When berry color was separated, yellow fruit was significantly different from the other three berry colors, although these were not significantly different from each other (Figure 2.2). Again, yellow raspberries took significantly longer to reach the bleed threshold than their red (*R. idaeus*) counterparts. Genotype analysis revealed that the two yellow genotypes took significantly longer to reach the threshold (Table 2.7).

Table 2.7: LS Means of disease incidence of field grown, primocane-harvested raspberry genotypes stored at 5°C in 2010 and 2011. Means followed by the same letter are not significant different (α=0.05).

Genotype	Species	Days to 25% Rot	Days to 25% Bleed
8FFBR	*R. x neglectus*	15.35 a	5.59 c
Caroline	*R. idaeus*	13.08 ab	6.74 c
Explorer	*R. occidentalis*	11.58 ab	5.61 c
Mandarin	*R. idaeus*	11.40 ab	5.04 b
Heritage	*R. idaeus*	10.34 ab	3.89 c
Prelude	*R. idaeus*	9.67 bc	6.10 c
Anne	*R. idaeus* (yellow)	6.61 c	11.63 ab
Kiwigold	*R. idaeus* (yellow)	5.68 c	12.66 a

2.3.3. Ethylene and Respiration

Ethylene evolution rates and respiration rates were measured on the following primocanes-fruiting raspberries: three red *R. idaeus* genotypes, one yellow *R. idaeus* genotype, one *R.* × *neglectus* genotype and one *R. occidentalis* genotype. Figures (2.4, 2.5, 2.6, and 2.7) were color-coded to match berry color. Day 1 measurements were made approximately 6 hours after harvest, after the fruit had been moved to the 5°C incubator. Ethylene evolution rates decreased from the first measurement to the second one. All four *R. idaeus* cultivars showed the ethylene response of traditional climacteric fruit both when unripe (Figure 2.4) and ripe (Figure 2.5). Explorer, the lone *R. occidentalis* genotype, did not show a climacteric response at either ripeness stage. 8FBBR, the only *R. x neglectus* genotype, does not show the characteristic climacteric curve when measured unripe, although ethylene evolution rates began to increase by day 7 in storage. When measured ripe, it had a suppressed ethylene-climacteric curve (Figure 2.5).

Respiration rates declined during the first days following harvest in all species for both ripe and unripe fruit (Figure 2.6, 2.7). No climacteric-like increase in respiration rate was detected in any of these cultivars.

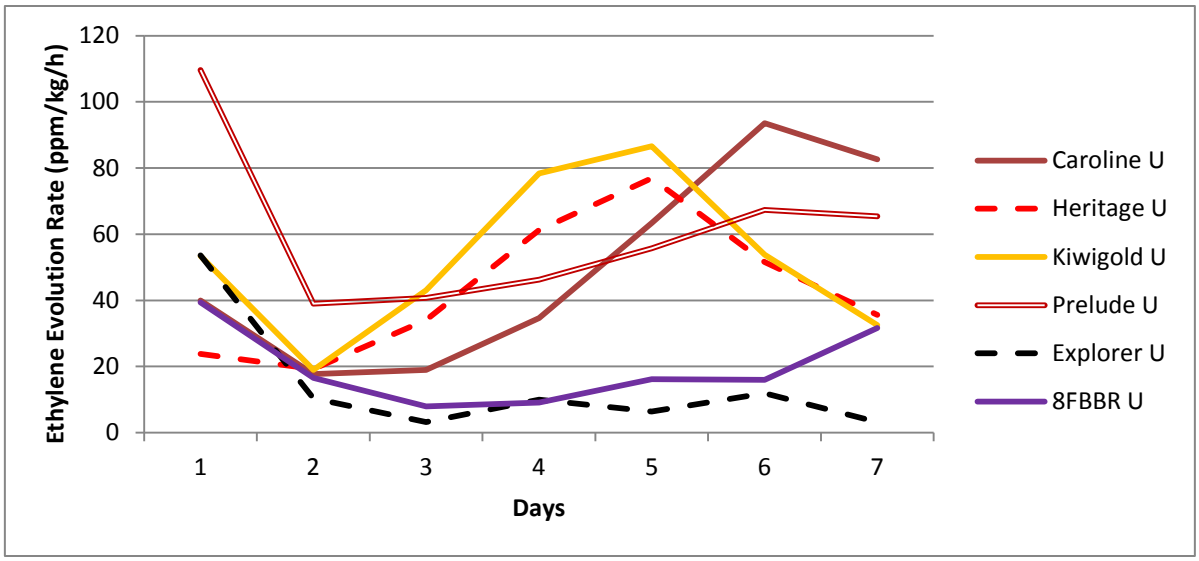

Figure 2.4: Ethylene Evolution Rates of Unripe (U), Primocane-Fruiting Genotypes measured for seven days in 2011.

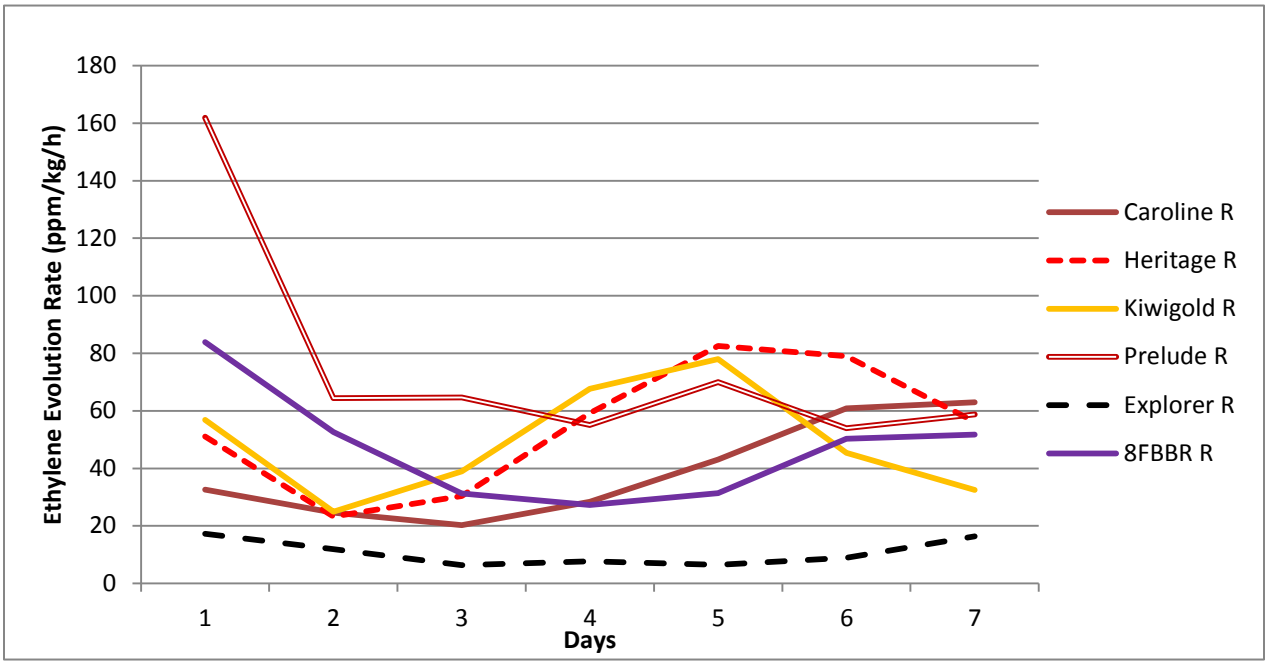

Figure 2.5: Ethylene Evolution Rates of Select, Ripe (R) Primocane-Fruiting Genotypes

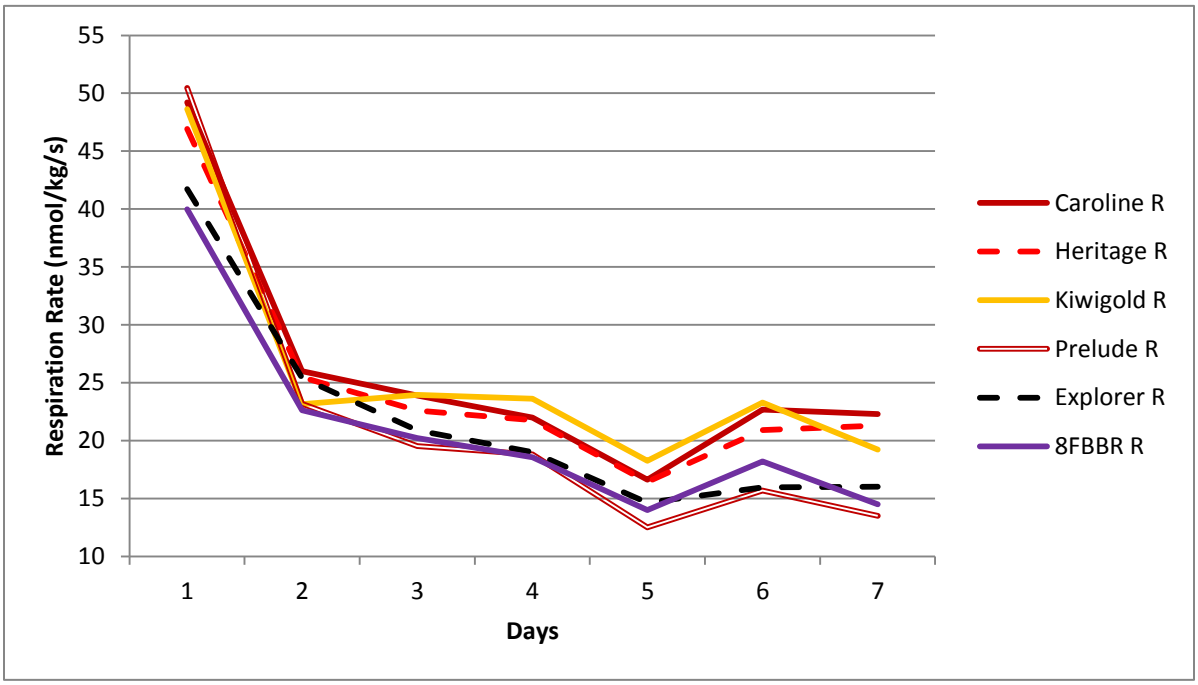

Figure 2.6: Respiration Rates of Select, Ripe (R) Primocane-Fruiting Genotypes

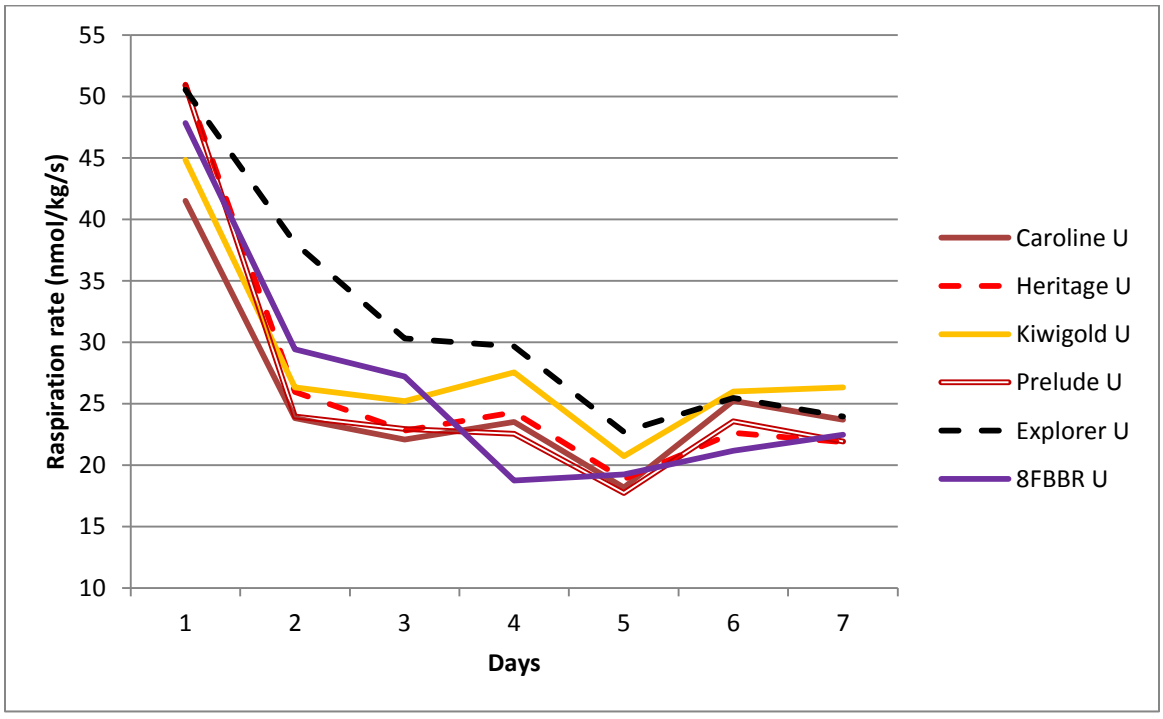

Figure 2.7: Respiration Rates of Select, Unripe (U) Primocane-fruiting Genotypes

2.3.4. Physiochemical Analysis

Physiochemical analysis of raspberry genotypes was performed over two years (2010 and 2011) for both harvest seasons (floricane and primocane). A minimum sample size of 400 g was needed for each analysis. After harvesting berries for storage tests, firmness measurements and inoculations, berries were harvested for physiochemical analysis. For that reason, not all of the plots included in the pathology study were analyzed. Notable omissions were Explorer, Munger and Mandarin.

In 2010, *R. occidentalis* had the highest phenolics and anthocyanins followed by *R.* × *neglectus* and then *R. idaeus* (Table 2.8). As expected, the two yellow cultivars, Anne and Kiwigold (*R. idaeus*), had the lowest levels of anthocyanins. The highest titratable acidity is seen in *R. idaeus* cultivars, followed by *R.* × *neglectus* and then *R. occidentalis.* Black raspberries (*R. occidentalis*) seemed to be the most firm. With the exception of Caroline (*R. idaeus*), the highest lipophilic ORAC values and hydrophilic ORAC values are seen in black raspberry genotypes (*R. occidentalis*) and purple raspberries (*R.* × *neglectus)* followed by red raspberries (*R. idaeus*).

Similar trends were seen in 2011, except that Spinefree Willamette (*R. idaeus*) and 8FBBR (*R.* × *neglectus*) also had very high phenolic and anthocyanin values (Table 2.9). Again, the two yellow cultivars had the lowest anthocyanins. In agreement with 2010 data, the highest titratable acidity is seen in *R. idaeus* genotypes, followed by *R.* × *neglectus* and then *R. occidentalis.* With the exception of Caroline and Spinefree Williamette (*R. idaeus*), the highest lipophilic ORAC values and hydrophilic ORAC values are seen in black raspberries (*R. occidentalis*) and purple raspberries (*R.* × *neglectus)* followed by red raspberries (*R. idaeus*).

Table 2.8: Average firmness and physiochemical variables across both harvest seasons in 2010. Units are expressed as the following: Newtons for Firmness (N), micromole Trolox equivalents per gram fresh weight for both lipophilic ORAC (L-ORAC) and hydrophilic ORAC (H-ORAC), as percent of citric acid equivalent for titratable acidity, and as milligrams Gallic acid equivalent for Anthocyanins and Phenolics.

Genotype	Species	N	L-ORAC	H-ORAC	TA	SSC	SSC:TA	Antho-cyanins	pH	Phenolic
Bristol	*occidentalis*	3.31	1.52	36.73	1.14	12.15	10.69	257.05	3.62	411.93
Huron	*occidentalis*	2.09	1.43	36.45	1.18	9.97	8.45	242.01	3.58	360.60
Jewel	*occidentalis*	1.83	1.33	35.30	1.23	10.93	7.68	369.54	3.61	397.24
NYO3-01	*neglectus*	1.59	1.00	27.22	1.34	9.35	7.01	71.51	3.23	204.84
8FFBR	*neglectus*	2.04	1.53	35.85	1.24	11.63	9.55	119.59	3.48	343.67
Royalty	*neglectus*	1.84	1.43	34.20	1.25	8.30	6.65	65.73	3.32	321.32
Anne	*idaeus*	1.33	0.95	26.77	1.38	11.78	8.60	1.42	3.22	201.55
Caroline	*idaeus*	0.99	1.30	34.62	1.56	9.75	6.23	47.95	3.08	290.74
Heritage	*idaeus*	1.95	0.82	21.41	1.51	11.15	7.39	27.36	3.00	208.70
Kiwigold	*idaeus*	1.80	0.93	25.53	1.50	10.53	7.03	1.38	3.14	213.84

Table 2.9: Average physiochemical variables across both harvest seasons in 2011. Units are expressed as the following: Newtons for Firmness (N), micromole Trolox equivalents per gram fresh weight for both lipophilic ORAC (L-ORAC) and hydrophilic ORAC (H-ORAC), as percent of citric acid equivalent for titratable acidity, and as milligrams Gallic acid equivalent for Anthocyanins and Phenolics.

Genotype	Species	L-ORAC	H-ORAC	TA	SSC	SSC:TA	Antho-cyanins	pH	Phenolic
Bristol	*occidentalis*	1.48	39.96	1.08	11.80	10.25	512.14	2.35	439.61
Earlisweet	*occidentalis*	1.41	36.10	1.30	12.00	11.32	300.85	3.28	398.26
Huron	*occidentalis*	1.44	36.73	1.21	10.18	8.45	283.60	2.97	358.09
Jewel	*occidentalis*	1.22	31.15	1.30	11.42	8.92	405.14	3.23	351.31
Wes-O4	*occidentalis*	1.26	31.98	1.60	7.97	5.40	234.48	4.08	320.38
8FFBR	*neglectus*	1.38	34.79	1.33	11.47	9.55	165.53	3.29	361.56
NYO3-01	*neglectus*	1.11	27.44	1.39	9.60	7.25	71.73	3.55	179.92
Royalty	*neglectus*	1.29	32.77	1.35	8.07	6.36	80.18	3.33	271.11
Anne	*idaeus*	0.98	24.37	1.41	11.07	8.60	0.87	3.55	174.89
Caroline	*idaeus*	1.32	33.40	1.57	10.00	6.23	40.88	3.80	281.30
Heritage	*idaeus*	0.95	23.95	1.52	11.08	7.39	45.47	3.67	203.79
Kiwigold	*idaeus*	1.15	27.44	1.52	10.50	7.03	1.36	3.71	210.77
Prelude	*idaeus*	1.12	27.67	1.50	10.83	6.42	38.81	3.67	244.97
Spinefree Willamette	*idaeus*	1.37	34.66	1.24	9.05	7.44	76.96	3.06	339.39

2.3.5. Multivariate Analysis of Physiochemical Variables for both 2010 and 2011.

Factor analysis was performed on the average disease incidence data for berries evaluated in cold storage and the physiochemical variables for both years Since ethylene and respiration measurements were done on only a limited number of harvest dates and for only a few of the plots, these measurements were not included. Firmness data were not included as only one year's data was taken.

The first two factors explained 69% of the variance and were selected for further analysis. Figure 2.8 shows that the number of days to 25% rot, total anthocyanin, total phenolics, and both hydrophilic and lipophilic antioxidant capacity cluster closely together. The other fruit quality measurements soluble solids content (SSC), titratable acids (TA), ratio of SSC to TA, and pH) did not cluster together. The number of days to 25% bleed did not cluster with the other variables. Table 2.10 shows the correlations between those variables. Total phenolic has the greatest correlation with rot incidence.

Table 2.10: Correlation of physiochemical variables and disease incidence variables that cluster together

	Days to 25% Rot	Total Anthocyanins	Total Phenolics	H-ORAC	L-ORAC
Days To 25% Rot	1.00	0.527 0.0002	0.72484 <0.0001	0.631 <0.0001	0.623 <0.0001
Total Anthocyanins	NR	1.00	0.828 <0.0001	0.646 <0.0001	0.581 <0.0001
Total Phenolics	NR	NR	1.00	0.883 <0.0001	0.85305 <0.0001
H-ORAC	NR	NR	NR	1.00	0.94935 <0.0001
L-ORAC	NR	NR	NR	NR	1.00

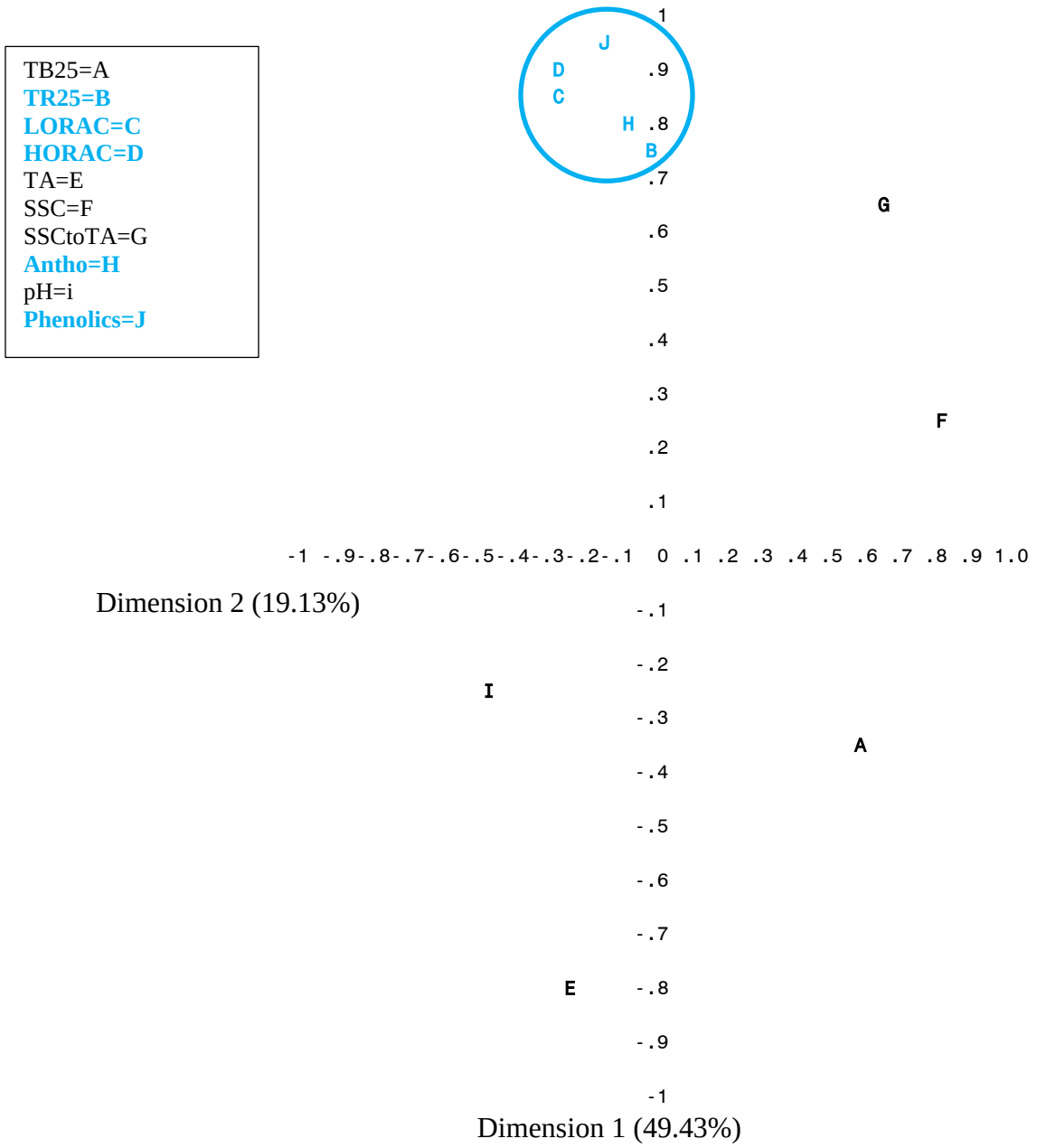

Figure 2.8: Factor Analysis Plot of Response Variables for 2010 and 2011.

2.3.6. Factor Analysis of Physiochemical Variables and Firmness in 2010

Firmness measurements were taken once a week in 2010. Those firmness measurements, disease incidence and physiochemical variables in 2010 were included in a second factor analysis. Factor 1 and 2 explained 68% of the variation, therefore, those two were chosen for analysis. Figure 2.8 shows that days to 25% rot, lipophilic antioxidant capacity, hydrophilic antioxidant capacity, total anthocyanins, total phenolics and pH cluster together, shown within the circle on the graph. Firmness lies near the ratio of SSC to TA. Days to 25% bleed, L*, a*, b* (color values) and titratable acids form a loose cluster, shown on the graph in a dotted circle. The cluster of these variables may reflect the close linkage of fruit quality measures and could be helpful in exploring how to reduce post-harvest bleed.

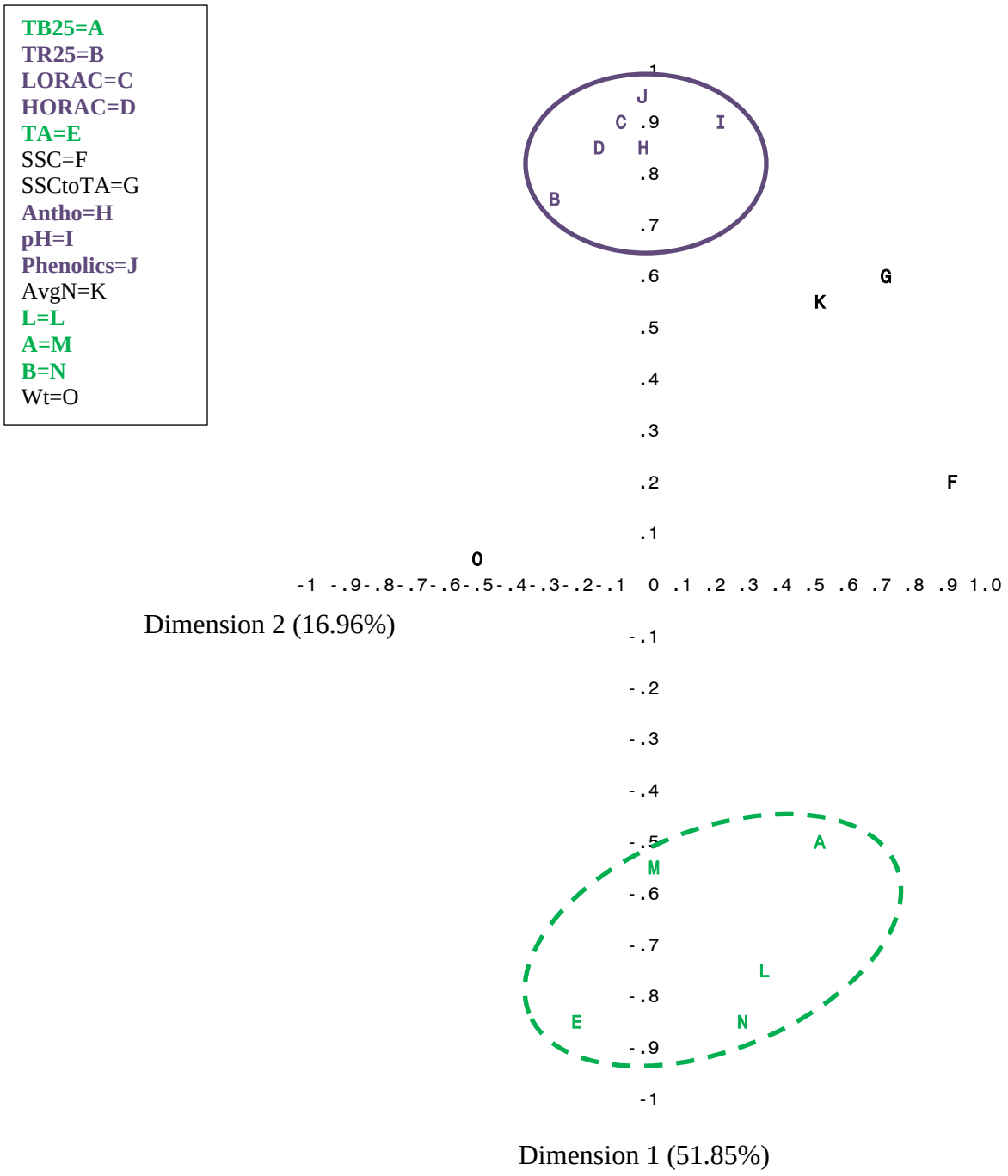

Figure 2.9: Factor Analysis Plot of Response Variables including Firmness Measures for 2010.

2.4. Discussion and Conclusion

Previous studies have extensively studied the post-harvest quality and physiological characteristics of red raspberries (*R. idaeus*). This study aimed at expanding that analysis to other commonly grown species of raspberries, focusing on post-harvest decay in an unsprayed field under conditions that mimic the capabilities of small producers of raspberries in the eastern United States.

Using warm storage (25°C) as a rapid, low input method for evaluating shelf-life of potential raspberry selections under heavy disease pressure did not yield the same information as storing them under cold storage (5°C) conditions. Species and berry color results were the same for warm and cold storage for species and berry color comparisons during the floricane harvest season when comparing rot incidence. All other seasons and comparisons within those seasons (species, berry color, genotype) were different. Warm storage should probably be abandoned as a low-input method for testing potential breeding selections. The rest of this discussion will focus on cold storage.

Fruiting season interacted significantly with measures of disease incidence, which is not surprising given that some of the cultivars that were evaluated differed between floricane and primocane cropping seasons. Raspberries tended to rot and bleed more quickly in the primocane-fruiting harvests than in the floricane-fruiting harvests. This phenomenon has been well documented in red raspberries and appears to be primarily due to increased rainfall coupled with greater inoculum in the field (Bristow 1991). For the floricane-fruiting season, black raspberries (*R. occidentalis*) and purple raspberries *(R. x neglectus)* resisted post-harvest decay the longest. Red raspberries and yellow raspberries had significantly shorter storageability. The ability of *R. occidentalis* to store

longer has been noted previously (Stephens et al. 2002), but susceptibility to other diseases has hindered their production and growth in the marketplace. For the primocane-fruiting season, only one black raspberry cultivar, 'Explorer,' and one purple raspberry breeding selection, '8FBBR' were available. Compared to two yellow *R. idaeus* cultivars and four red *R. idaeus* cultivars, that particular black raspberries stored significantly longer than all other colors of raspberries. 8FBBR (*R. x neglectus*) and the red *R. idaeus* did not differ significantly from one another. If Explorer is a genotype representative of *R. occidentalis* and 8FBBR is a genotype representative of *R.* × *neglectus*, then black raspberries also resist post-harvest rot longer when fruiting on primocanes.

Rot incidence and bleed incidence were not the same; the species, berry colors and genotypes that performed well in regards to rot were not the same that performed well in regards to bleed. The genotypes that did well in at least one of the harvest seasons for rot included Munger, Bristol, Explorer, Spinefree Willamette, 8FBBR and Caroline. The genotypes that did well for bleed incidence included Kiwigold, Anne, and Mandarin. It appears that bleeding is not a response associated with fungal infection and decay. It may be governed by other physiological mechanisms such as membrane degeneration in storage or damage sustained during harvest.

The decrease of ethylene measured between the first measurement at six hours to the second, a day later, is likely to reflect a wound response following harvest coupled with equilibration to 5°C. This pattern occurred in both ripe and unripe berries. Respiration rates showed the same initially higher measurement and decline, probably due to the slow loss of field heat prior to equilibrating 5°C.

Ethylene evolution and respiration rates were in agreement with the current literature. *R. idaeus* and *R.* × *neglectus* raspberries have an ethylene evolution peak characteristic of climacteric fruit, while their respiration rates are characteristic of non-climacteric fruit (Perkins-Veazie and Nonnecke 1992, Iannetta et al. 1999, Burdon and Sexton 1990). Interestingly, the relative ethylene evolution rates of the different genotypes agreed with the incidence of rot data. Black raspberries (*R. occidentalis*) evolved the least amount of ethylene and inversely took the longest to reach the disease threshold. Purple raspberries (*R.* × *neglectus*) evolved more ethylene than the black raspberries but less than the red or yellow raspberries, which also agreed with their storage data—purple raspberries resist postharvest infection longer than red or yellow raspberries, but less than black raspberries. This was also seen in several additional post-harvest trials (data not shown).

This observation is puzzling when compared to what is known about the relationship between *B. cinerea* and plants. Diaz suggested that plant-produced ethylene antagonizes the fungus and limits its growth (Diaz et al. 2002). However, that study focused on ethylene that was produced by the leaf. Work done with tomato fruit shows that *B. cinerea* can activate some ripening-related genes, potentially making it easier for the fungus to infect (Cantu et al. 2009). In this study, the fruit were all picked at the same degree of ripeness and yet, those raspberry fruit producing the most ethylene are also the genotypes that succumbed to post-harvest rot most quickly. Diaz' results would suggest that red raspberries should be the most resistant to *B. cinerea* since they are evolving the greatest amount of ethylene. Clearly this is not the case and other physiological difference between the genotypes, such as thickness of epidermis or of waxy cuticle,

could be the cause. Anthocyanin or phenolic content may also play a role as these are also higher in the black and purple raspberries.

Our results comparing the incidence of bleed in storage among genotypes and species offer less straightforward conclusions. When grouping red and yellow within species, the difference between yellow and red is lost. Yellow raspberries take the longest amount of time to reach the bleed threshold possibly due to the difficulty in seeing the juice against the white filter paper. Red raspberries took significantly longer time to reach the threshold when compared to black and purple raspberries when fruiting on floricanes. Only yellow raspberries were significantly different when fruiting on primocanes. As shown by the factor analysis, color values and titratable acidity clustered with our bleed measurements. This suggests that there is some additional physiological mechanism that controls post-harvest bleed, but it is unclear what that is as it was not a focus of this study. What does appear to be clear is that the mechanisms controlling bleed and the mechanisms controlling resistance to decay were distinct.

Factor analysis revealed that several chemical properties (ie. anthocyanin concentration, total phenolics, hydrophilic and lipophilic ORAC values) cluster together with fruit resistance to rot. There is the potential for new cultivars to be developed that are both healthier for the consumer and simultaneously better for the grower. Purple raspberries (*R.* x *neglectus*) offer the opportunity of joining the plant disease resistance of red raspberries (*R. idaeus*) with the extended storageability and presumed greater health benefits of black raspberries (*R. occidentalis).* It is unclear whether a phenotypically red *R. x neglectus* could be created that would maintain the storageability and health benefits

without their increased anthocyanins. Further studies and breeding are certainly needed to investigate this potential opportunity.

Chapter 3: Further Discussion

3.1. Conclusions and Greater Importance

In hindsight, I should have pursued observations from 2010 that *R. stolonifer* differentially infected these cultivars. Those results should have been made a greater research priority. Furthermore, differential infection by this fungus may have explained the differences in time to 25% bleed that was observed. Yellow raspberries are the berry color most affected by this. They could be succumbing to *B. cinerea*, which would explain the time to 25% rot to be relatively short, but highly-resistant to *R. stolinifer*, which might explain the time to 25% bleed to be relatively long.

Ethylene evolution rates observed and the incidence of decay data do not agree with some published studies. Later in the season, *Rhizopus stolinifer*, a lesser, common post-harvest disease was seen more readily, especially at 25°C. It could be possible that ethylene has a stimulating effect, rather than antagonistic effect, on this fungus. Berries infected with *R. stolinifer* were counted as infected, perhaps clouding the response of raspberry species or cultivars and *B. cinerea*. More work should have been done to sort out the effect of *R. stolinifer*, *B. cinerea* and storage temperature.

Regardless of the reason, ethylene evolution rates and rot incidence are inverse. It is interesting that in more than 30 years of post-harvest research the mechanism of fruit ripening in raspberries is still debated. Is an ethylene peak that occurs late in ripening enough to consider it climacteric fruit? Or does the lack of concomitant rise in respiration mean it is a non-climacteric fruit? Due to raspberry and the genus *Rubus* representing such a small portion of the commercial produce industry, far more work has been done in other crop ripening systems such as tomato, apple and peach. Several discoveries have

been made in the past ten years that shed more light on the ripening mechanism of all fruits. The first one is that there are two systems of ethylene production in the plant. System 1 is the basal, auto-inhibitory ethylene pathway. System 2 is the auto-catalytic pathway associated with fruit ripening. The switch from System 1 to System 2 is under genetic control and is being investigated currently. There are also multiple ACS genes in tomato that appear to segregate to one of the two systems, or control the switch from System 1 to System 2. It has also been shown in tomatoes that there is an ethylene independent developmental system. Even when System 1 is eliminated, the fruit can still transition to System 2 and ripen (Paul, Pandey and Srivastava 2012). This could be one of the reasons that raspberries may only show System 2 ripening later in their development. Perhaps the ripening that occurs during less-mature stages is under the control of an ethylene- independent system.

Mango fruits show two ethylene peaks, one during early ripening and one later in ripening. Research has shown that the rate of fruit ripening in mango is correlated with external anatomical features, such as epidermis thickness, cuticle and wax presence and thickness, lenticel number, cell size and surface to volume ratio of cells. These characteristics affect the internal environment by changing the gas permeability of the fruit. Respiration requires oxygen, so a preferential permeability of a fruit could slow the rate of respiration without reducing ethylene synthesis. Permeability could also play a role in raspberry ripening. To reduce water loss after harvest, a less permeable or selectively permeable epidermis/cuticle might be adaptive for that crop (Paul et al. 2012).

Another interesting concept related to fruit during ripening is a switch to cyanide-resistant respiration. Cyanide is produced by some seeds as well as a byproduct of the

ACC oxidase pathway. If the cellular pH is near or above 7, and rate of diffusion of the gaseous toxin and detoxification mechanisms are slow, then the plant can switch to cyanide-resistant respiration. This is a far less efficient form of respiration, but it may explain why no concomitant spike in respiration was observed in raspberry as the fruit ripens. If the spike in ethylene is causing cyanide concentrations to rise greatly, possibly exacerbated by a hypothetical selective permeability of the fruit skin, then a switch to this secondary respiration pathway could conceivably occur (Millenaar and Lambers 2003).

3.2. Future Studies

The most interesting result came through the factor analysis that combined ORAC variables and storage variables. With additional study, I would like that graph to include all of these variables plus firmness, fresh weight, ethylene and respiration rates. With that information, the relationship between those variables and resistance to rot could be clearly elucidated. The original goal was to determine the effect of storage temperature on the ability to detect differences in disease incidence among species, berry colors, and genotypes. The results show that there are differences in disease incidence between species, berry colors and genotypes. Following from that, what particular species, berry colors, genotypes are responsible for that difference? If I had measured all the variables listed above in a way that they could be combined, we would gain a better understanding of the linkage between physiology and pathology. This would allow the next researcher to continue this thread to design specific experiments to test those significant variables.

Another study that I had wanted to conduct was the comparison of the ethylene synthesis and ethylene receptor genes during the development of raspberry fruits and their receptacles. One recently-published paper looked at ACC synthase in ripening in

raspberry fruit and concluded that it resembled a non-climacteric fruit (Zheng and Hrazdina 2010b). Similar work done in peaches, which are climacteric, showed the change in expression level of different ethylene receptors (Rasori et al. 2002). Using their work as a template, I would like to use two cultivars representative of each berry color (and species) at four different ripening stages using fruit and their receptacles to study the expression levels of ethylene synthesis genes, ACC oxidase and AAC synthase, and receptor genes, ETR1 and ERS1. ETR1's expression levels should remain unchanged throughout ripening as it is constitutive, while ERS1's expression levels would be expected to increase. Other ethylene receptors could eventually be added if this preliminary work is promising. While not proving whether raspberries are climacteric fruits, it would add another piece to the puzzle. This fruit clearly differs from well-studied model fruits such as apple and tomato.

3.3. Hindsights

One of the most challenging things I encountered during the planning, execution and analysis of this project was my own zealousness. Originally, this project originally contained the following objectives: physical measurements (firmness, color, GC data, weight), pathological analysis (storage data, inoculations, and ethylene/*B. cinerea* interaction experiments), and genetic expression (real time-PCR of ethylene biosynthesis genes). Because I tried to do too much, a lot of it ended up not getting done or not getting done well enough to facilitate a straight forward data analysis process. The ethylene/ *B. cinerea* interaction experiments and the genetic analysis were not begun. Due to equipment failure and the time consuming nature of the GC analyses, those were only analyzed four times during the two years of this thesis work.

The storage data, which should be the lynchpin of my entire thesis, suffered greatly from the time that was spent on the other aspects of the project. Data were taken every 2-3 days rather than daily. To analyze these data, I had to interpolate the time to 25% rot and bleed. As I wanted to do everything, things were not done as well as they could have been. In my future research projects, I will need to be more careful when budgeting my time and being more realistic about what I can get done in a day.

One of the most challenging things to overcome in data analysis was the original field layout and cultivar availability, which was designed for a different study to test response to virus. The field is currently blocked with only one plot of each genotype in each block. A better design would have been to replicate each cultivar plot at least twice, ideally three times, in each block. This would have allowed me more degrees of freedom when using ANOVA and more accuracy for all of my data. I would have also chosen a location without dramatic soil differences—walking through the field and aerial photographs show the gradient of soil. Differences in soil within this planting could be confounding my results. Since the soil differences in this plot occur on a perpendicular axis, it makes the appropriate blocking particularly challenging. As for genotype availability, there were not enough *R. x neglectus* and *R. occidentalis* genotypes available that are primocane-fruiting to completely test my hypothesis, at the time of its planting in 2007.

In the experimental design, I would have chosen at third temperature (-0.5°C) in addition to 5°C and 25°C. While the data from the 25°C treatments was not useable for meaningful analysis, it did show me that using room temperature storage as the first test of a new selection's shelf-life was not the best approach. Instead I should have used

-0.5°C, as that is the shipping-industry standard. Adding that temperature would also have given a more complete picture of resistance to disease in storage, and would have allowed me to also have more applicable results for the fresh-fruit shipping industry.

Firmness measurements began in June of 2010, just a few months after completing my undergraduate degree. Without a proper review of the literature at that time, we decided on a measurement of firmness that is akin to crushing. Berries were placed receptacle-cavity down on the plate of a texture analyzer. A wide, flat, steel circle would press down on the berry. The force necessary to crush the berry was recorded and that was our measure of firmness. After reviewing the literature on fruit morphology, the standards for measuring firmness in raspberries were very different from what was measured. The standard measure of whole berry firmness is done by placing the berry on its side and measuring the amount of pressure required to close the receptacle cavity. This appears to be a better approximation of a berry's ability to withstand shipping. Another measure of firmness in raspberries is the force required to puncture the drupelet. This would have been a good measure of the berry's ability to withstand pathogen and harvest injury. Differences in the way that raspberries are measured for firmness may be the cause of any conflicting results of this study and previous literature.

Coursework was another hindrance to completing my project. While I eventually completed three semesters of statistics, my first season's data was taken before any of those courses. In hindsight, the biggest hindrance was not taking the storage data daily. The data I was taking was the raw number of berries each time I took data that were showing bleed or rot. I calculated proportion of berries showing postharvest decay incidence. As I didn't have those proportions for each day for every plate, they couldn't

be compared directly. I had to interpolate and calculate the 25% variable to remedy this problem. I staggered the days on which I took additional measurements (inoculations, firmness) and I should have taken as many variables in the same day as possible, to avoid confounding. There are also no plant breeding courses offered that would have fulfilled my need for a better understanding of classical genetics and programs used by current plant breeders to calculate the degree of relatedness among cultivars and species, which would have been useful.

The importance of reliable equipment was made painfully clear to me in the second harvest year. Multiple equipment failures occurred—cold room temperature controls failed causing several cold treatment replications to be discarded, and the home-made auto-sampler, assembled by a now-retired scientist, failed. Consequently, some data were missed. At that point my respiration and ethylene protocols were changed. Sharing equipment with multiple people also had major drawbacks. The texture analyzer equipment and software was being used by others and was not easily reset. Due to that, my second year's firmness data was not reliable and was eventually discarded. Sharing issues also meant that the colorimeter was not always available. This comedy of failures made real the scientific process in a different way—every experiment does not run smoothly, results are not always as expected, and the importance of reliable equipment cannot be overstated!

Appendices

Appendix A: Inoculations

Materials and Methods

For selected harvests, berries were harvested using the identical sterile techniques and then sterilized with a 70% ethanol solution. *B. cinerea* cultures were maintained on raspberry agar plates and potato dextrose agar plates. Growth was staggered to ensure available conidia when needed. Spores were harvested by washing the plates with 2-3mL of sterilized distilled water containing Tween20 (1µL/1mL water.) The spore suspension was quantified using a hemacytometer and adjusted with sterile distilled water. The berries were placed in 12-well Costar plates and surface inoculated with 10 µL 10^4 condiospore suspension of *B. cinerea.* Plates were then incubated at 5°C and disease incidence was recorded for two weeks. If there were no signs of *B. cinerea* at the end of two weeks, the plates were moved to room temperature to ensure the applied inoculum was viable.

Results

The data yielded from inoculations performed in 2010 was inadequate for determining if there were any differences in the varietal susceptibility to *B. cinerea.* The protocol was adjusted in 2011 to allow for longer time to observe the berries. Analysis of variance performed on the 2011 data revealed that harvest date is significant ($P>0.0001$). Species was not significant for time to 25% rot. As there were only 7 genotypes and a total of 34 observations, this might be too little data to be statistically meaningful. The same results are observed for berry color.

Appendix B. Weather Data

Weather data were obtained from a continuously running weather station located outside of the research plot. Environmental variables obtained included minimum and maximum temperature, minimum and maximum humidity, average radiation and precipitation. Averages from those data were calculated for the three days prior to harvest. As raspberries were harvested very early in the morning, weather data from the day before harvest was used. As in storageability tests, statistical analysis split the weather by fruiting season due to the known interaction of season on the storageability of species.

Weather data prior to harvest was largely inconclusive (Table D.1). Total precipitation for the three days prior to harvest was significant for three out of the four tests. It explained the majority of variation in the floricane-harvests for rot incidence, but in the primocane-harvests, it explained only 1.96% of the variation. None of the other variables were significant for both harvest seasons for the same measure of storageability.

Table B.1: Stepwise regression weather model data with R^2 values and (p-values). Only values that remained in the final model for each measure of disease incidence are shown.

	Floricane Harvests		Primocane Harvests	
	Average to 25% Rot	*Average to 25% Bleed*	*Average to 25% Rot*	*Average to 25% Bleed*
Precipitation, BH	22.51% (0.01)	----------	1.96% (<0.0001)	12.36% (0.0031)
Precipitation, DB	----------	----------	2.59% (0.0233)	11.39% (<0.0001)
Average Radiation, DB	13.63% (NS)	----------	----------	13.22% (NS)
Max Temp, BH	12.84% (0.04)	----------	----------	----------
Max Temp, DB	11.24% (0.035)	----------	----------	----------
Minimum Temp, DB	----------	----------	----------	12.9% (0.0260)
Max Humidity, DB	----------	----------	----------	9.13% (<0.0001)
Min Humidity, DB	20.22% (0.005)	----------	----------	----------
Min Humidity, BH	17.43% (0.0013)	8.61% (<0.001)	----------	----------

Note: BH: three days before harvest, DB: day before, (------) not included in the model due to lack of significance.

Appendix C. Genetic Identification of *Rhizopus stolinifer*

A second fungus that commonly appeared on the raspberries undergoing storage tests was cultured from symptomatic fruit and maintained on PDA. There was difficulty identifying the fungus using morphological characteristics as either *Rhizopus stolinifer* or *Mucor piriformis,* so genetic identification was pursued using the intertranscribed ribosomal RNA spacer.

Mycelial mats were cultured in PDB liquid shake cultures at 150rpm in incubators at 25°C. Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) following the manufacturer's protocol. The quantity and quality of the template was assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). The complete nucleotide sequence for the ITS (intertranscribed ribosomal RNA spacer) locus was obtained from *Rhizopus stolonifer* GenBank accession DQ767605.1. The following, gene specific primer set was used to perform conventional PCR: Forward- 5’ GAT TGG GCA AAG TCT TGG TT 3’; Reverse- 5’ TTC TTT GTT CTC TCC CTT CTT CA 3’. The PCR cycling parameters were as follows: initial denaturation for 3 min at 95°C; 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were loaded on a 1% TBE agarose gel with containing ethidium bromide and electrophoresed at 100 V for 50 min according to standard procedures as outlined in Sambrook and Russell, 2001. Bands on the gel were visualized using an Alpha imager mini UV light box (Model M-26E; Alpha Innotech Corporation). PCR amplicons were purified using QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA). The PCR products were then sequenced directly by Macrogen Inc. (Rockville, MD) using the

Sanger method and the gene specific primers stated above. Fungal identification of the *Rhizopus stolinifer* isolate was carried out at the genus and species level by sequencing the ITS2 locus.

Appendix D. SAS Codes

```
PROC MIXED DATA=FLORICANE;
CLASS YEAR REP PLOT SPECIES
TEMP;
MODEL TR25= SPECIES | TEMP /
DDFM=SAT;
RANDOM REP YEAR;
REPEATED / SUBJECT=PLOT;
LSMEANS SPECIES*TEMP
/adjust=tukey;
RUN;

PROC MIXED DATA=FLORICANE;
CLASS YEAR REP PLOT SPECIES
TEMP;
MODEL TB25= SPECIES | TEMP /
DDFM=SAT;
RANDOM REP YEAR;
REPEATED / SUBJECT=PLOT;
LSMEANS SPECIES*TEMP;
RUN;

PROC MIXED DATA=FLORI5;
CLASS YEAR REP PLOT;
MODEL TR25= SPECIES;
REPEATED / SUBJECT=PLOT;
RANDOM REP YEAR;
LSMEANS SPECIES / ADJUST=TUKEY;
RUN;

PROC MIXED
DATA=FLORICANECOLD4SP;
CLASS YEAR REP PLOT SPECIES;
MODEL TR25= SPECIES;
REPEATED / SUBJECT=PLOT;
RANDOM REP YEAR;
LSMEANS SPECIES / ADJUST=TUKEY;
RUN;


PROC FACTOR DATA=ORAC NFACTORS=2
PLOT EIGENVECTORS OUT=D;
VAR TB25 TR25 LORAC HORAC TA SSC
SSCtoTA Antho pH Phenolics;
RUN;

PROC FACTOR DATA=ALL
EIGENVECTORS PLOT;
VAR TB25 TR25 LORAC HORAC TA SSC
SSCtoTA Antho pH Phenolics avgn
L A B wt;
RUN;
```

Appendix E. Useful Diagrams

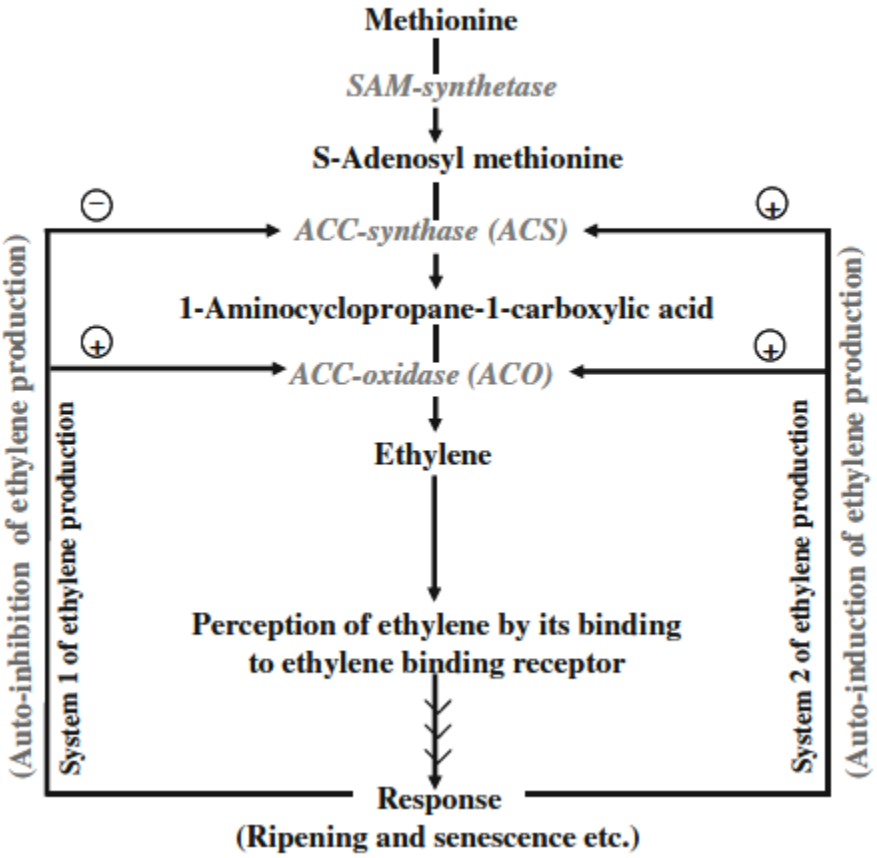

Figure E.1: Simplified Ethylene Biosynthesis Pathway (Paul et al. 2012)

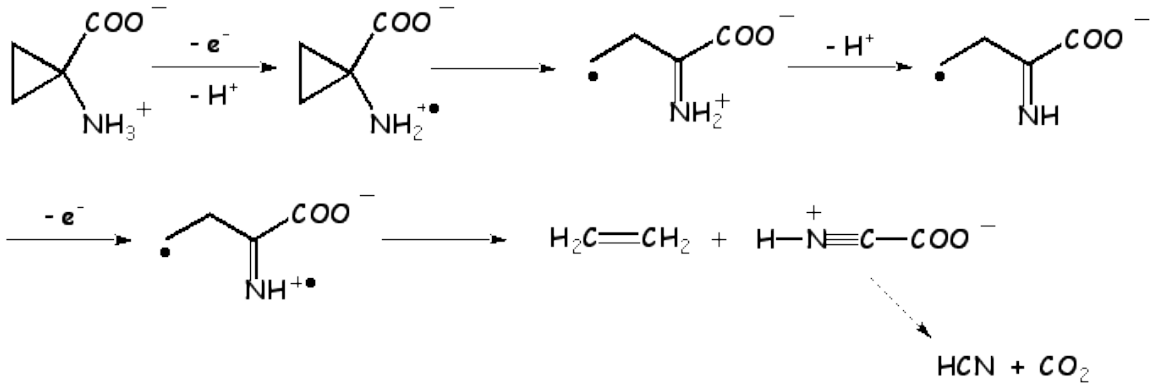

Figure E.2: ACC oxidase Evolves Cyanide

References

2002. Overview of the Raspberry and Blackberry Industry. The North American Raspberry & Blackberry Association.

2007. *Rubus* L. USDA, ARS, National Genetic Resources Program.

Aprea, E., S. Carlin, L. Giongo, M. Grisenti & F. Gasperi (2010) Characterization of 14 Raspberry Cultivars by Solid-Phase Microextraction and Relationship with Gray Mold Susceptibility. *Journal of Agricultural and Food Chemistry,* 58**,** 1100-1105.

Blanpied, G. D. (1972) Study of Ethylene in Apple, Red Raspberry, and Cherry. *Plant Physiology,* 49**,** 627.

Bristow, P. R. 1991. Fruit and Flower Diseases Caused by Fungi: Botrytis Fruit Rot (Gray Mold) and Blossom Blight. In *Compendium of Raspberrry and Blackberry Diseases and Insects,* eds. M. A. Ellis, R. H. Converse, R. N. Williams & B. Williamson. St. Paul, Minnesota: APS Press.

Burdon, J. N. & R. Sexton (1990) Fruit Abscission and Ethylene Production of Red Raspberry Cultivars. *Scientia Horticulturae,* 43**,** 95-102.

Cantu, D., B. Blanco-Ulate, L. Yang, J. M. Labavitch, A. B. Bennett & A. L. T. Powell (2009) Ripening-Regulated Susceptibility of Tomato Fruit to Botrytis cinerea Requires NOR But Not RIN or Ethylene. *Plant Physiology,* 150**,** 1434-1449.

Celik, M., T. Kalpulov, Y. Zutahy, S. Ish-shalom, S. Lurie & A. Lichter (2009) Quantitative and qualitative analysis of Botrytis inoculated on table grapes by qPCR and antibodies. *Postharvest Biology and Technology,* 52**,** 235-239.

Davis, R. P. 1991. Fruit and Flower Diseases Caused by Fungi:Postharvest Soft Rot (Leak Disease). In *Compendium of Raspberrry and Blackberry Diseases and Insects,* eds. M. A. Ellis, R. H. Converse, R. N. Williams & B. Williamson. St. Paul, Minnesota: APS Press.

Diaz, J., A. ten Have & J. A. L. van Kan (2002) The role of ethylene and wound signaling in resistance of tomato to Botrytis cinerea. *Plant Physiology,* 129**,** 1341-1351.

Dossett, M., N. V. Bassil, K. S. Lewers & C. E. Finn (2012) Genetic Diversity in wild and cultivated black raspberry (*Rubus occidentalis* L.) evaluated by simple sequence repeat markers. *Genetic Resources and Crop Evolution,* 59.

Dossett, M., J. Lee & C. E. Finn (2008) Inheritance of phenological, vegetative, and fruit chemistry traits in Black raspherry. *Journal of the American Society for Horticultural Science,* 133**,** 408-417.

Dossett, M., J. Lee & C. E. Finn (2010) Variation in anthocyanins and total phenolics of black raspberry populations. *Journal of Functional Foods,* 2**,** 292-297.

Fennell, A. 2012. Raspberry Culture.

Geisler, M. 2012. Agricultural Marketing Resource Center - Raspberries.

Iannetta, P. P. M., J. van den Berg, R. E. Wheatley, R. J. McNicol & H. V. Davies (1999) The role of ethylene and cell wall modifying enzymes in raspberry (Rubus idaeus) fruit ripening. *Physiologia Plantarum,* 105**,** 338-347.

Jarvis, W. R. (1962) Infection of Strawberry and Raspberry Fruits by Botrytis cinerea Fr. *Annals of Applied Biology,* 50**,** 569-&.

Kruger, E., H. Dietrich, E. Schopplein, S. Rasim & P. Kurbel (2011) Cultivar, storage conditions and ripening effects on physical and chemical qualities of red raspberry fruit. *Postharvest Biology and Technology,* 60**,** 31-37.

Le Couteur, P. & J. Burreson. 2004. Napolean's Buttons: 17 Molecules that Changed History. New York, NY: Penguin Group.

Lee, J., M. Dossett & C. E. Finn (2012) Rubus fruit phenolic research: The good, the bad, and the confusing. *Food Chemistry,* 130**,** 785-796.

Michael, D., N. V. Bassil, K. S. Lewers & C. E. Finn (2012) Genetic Diversity in wild and cultivated black raspberry (*Rubus occidentalis* L.) evaluated by simple sequence repeat markers. *Genetic Resources and Crop Evolution,* 59.

Millenaar, F. F. & H. Lambers (2003) The alternative oxidase: in vivo regulation and function. *Plant Biology,* 5**,** 2-15.

Owino, W. O. & H. Ezura. 2008. Ethylene Perception and Gene Expression. In *Postharvest Biology and Technology of Fruits, Vegetables and Flowers,* eds. G. Paliyath, D. P. Murr, A. K. Handa & S. Lurie. Ames, Iowa: Wiley-Blackwell Publishing.

Paliyath, G. & D. P. Murr. 2008. Biochemistry of Fruit. In *Postharvest Biology of Fruits, Vegetables and Flowers,* eds. G. Paliyath, D. P. Murr, A. K. Handa & S. Lurie. Ames, Iowa: Wiley-Blackwell Publishing.

Paul, V., R. Pandey & G. C. Srivastava (2012) The fading distinctions between classical patterns of ripening in climacteric and non-climacteric fruit and the ubiquity of ethylene-An overview. *Journal of Food Science and Technology-Mysore,* 49**,** 1-21.

Perkins-Veazie, P. & G. Nonnecke (1992) Physiological changes during ripening of raspberry fruit. *HortScience,* 27**,** 331-333.

Quidde, T., P. Buttner & P. Tudzynski (1999) Evidence for three different specific saponin-detoxifying activities in Botrytis cinerea and cloning and functional analysis of a gene coding for a putative avenacinase. *European Journal of Plant Pathology,* 105**,** 273-283.

Rasori, A., P. Tonutti, A. Ramina, B. Ruperti & C. Bonghi (2002) Characterization of two putative ethylene receptor genes expressed during peach fruit development and abscission. *Journal of experimental botany,* 53**,** 2333-2339.

Reeve, R. M. (1954) Fruit Histogenesis in *Rubus strigosus*. 1. Outer Epidermins, Parenchyma, and Receptacle. *American Journal of Botany,* 41**,** 152-160.

Robbins, J., T. M. Sjulin & M. Patterson (1989) Postharvest Storage Characteristics and Respiration Rates in 5 Cultivars of Red Raspberry. *Hortscience,* 24**,** 980-982.

Robbins, J. A. & J. K. Fellman (1993) Postharvest physiology, storage and handling of red raspberry. *Postharvest News and Information,* v. 4(2) p. 53N-59N.

SLJ. 2011. Orange Rust. In *Cornell Plant Disease Diagnostic Clinic*.

Staples, R. C. & A. M. Mayer (1995) Putative Virulence Factors of *Botrytis cinerea* Acting as a Wound Pathogen. *Fems Microbiology Letters,* 134**,** 1-7.

Stephens, M. J., P. Alspach, H. K. Hall & C. Kempler. 2002. Red Raspberry- Grey Mould Resistance from Rubus Species. Acta Hort (ISHS).

Stevens, F. L. (1914) A Destructive Strawberry Disease. *Science,* 39.

Tallman, P. 2009. Rubus occidentalis L. 'Explorer'. GRIN.

Tosun, M., M. Sengul, H. Karlidag & S. Ercisli (2009) Characterization of Red Raspberry (Rubus idaeus L.) Genotypes for Their Physicochemical Properties [electronic resource]. *Journal of food science an official publication of the Institute of Food Technologists,* 74**,** c575-c579.

Weber, C. A. (2003) Genetic diversity in black raspberry detected by RAPD markers. *Hortscience,* 38**,** 269-272.

Zheng, D. & G. Hrazdina (2010) Cloning and characterization of an expansin gene, RiEXP1, and a 1-aminocyclopropane-1-carboxylic acid synthase gene, RiACS1 in ripening fruit of raspberry (Rubus idaeus L.) [electronic resource]. *Plant Science,* 179**,** 133-139.