Induced defenses in apple fruits: linking fruit chemistry, quality, and plant-insect-microbe interactions

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ABSTRACT

Plants synthesize a diverse array of phytochemicals in response to interactions with herbivores, pathogens, and commensal microbes. These phytochemicals may simultaneously enhance crop defense and quality, representing a potential pest management strategy. However, plant chemical responses to different types and levels of biotic interactions remain unclear, particularly in fruit tissues, and the feasibility of inducing these defenses through elicitor application in field environments also requires further examination. Thus, apples were used to 1) examine the impact of distinct communities of biotic interactions among plants, insects, and microbes on fruit phenolic chemistry, and 2) examine the impact of the phytohormones jasmonic acid (JA), salicylic acid (SA), and melatonin (M) on fruit phenolic chemistry and resistance against pests and pathogens. Ultimately, phenolic defenses were induced by fungal damage primarily in ripe pulp tissues, where there was also a positive relationship between fungal endophyte and phenolic diversity, supporting a broad hypothesis that chemical diversity may increase with biotic diversity. Additionally, two compounds were upregulated in response to fungal damage: chlorogenic acid and an unidentified benzoic acid. Elicitor applications did not affect phenolic chemistry, but the combined application of JA-SA analogues had some chemical or physical effect, as this treatment reduced emergence of the insect *Rhagoletis pomonella*. Thus, fruit induced defenses may be tissue-specific and subject to temporal, environmental, or genotypic variation. Overall, these chapters examined the relationship between biotic interactions and induced fruit chemistry, with the goal of improving understanding of plant-microbe-insect interactions and incorporating these interactions into more sustainable agricultural practices.

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GENERAL AUDIENCE ABSTRACT

Plants may produce a diverse array of defensive phytochemical compounds in response to interactions with herbivores, pathogens, and the microorganisms that reside within plant tissues. These phytochemicals may simultaneously improve crop defenses and quality, representing a potential agricultural management strategy. However, plant chemical responses to different types and levels of biotic interactions are not well-understood, particularly in fruit tissues, and the feasibility of activating these defenses in fruits through the application of phytohormones that regulate defense pathways as a potential management strategy also requires further examination. Thus, apples were used to 1) examine the impact of distinct communities of biotic interactions among plants, insects, and microbes on fruit chemistry, focusing on phenolics, an important class of phytochemical compounds, and 2) examine the impact of the defense-activating phytohormones jasmonic acid (JA), salicylic acid (SA), and melatonin (M) on fruit phenolic chemistry and resistance against pests and pathogens. Ultimately, phenolic defenses were activated by fungal damage primarily in ripe pulp tissues, where there was also a positive relationship between fungal endophyte and phenolic diversity, supporting a broad hypothesis that chemical diversity may increase with biotic diversity. Additionally, two compounds were produced in response to fungal damage: chlorogenic acid and an unidentified benzoic acid. Phytohormone applications did not affect phenolic chemistry, but the application of the combined JA-SA analogues had some chemical or physical effect, as this treatment reduced emergence of the insect *Rhagoletis pomonella*. Overall, the phytochemical defenses activated by biotic interactions in fruits may occur primarily in certain tissue types, and may also vary due to environmental conditions, time of year, or plant species. These chapters examined the relationship between fruit chemistry and biotic interactions with the goal of improving understanding of plant-microbe-insect interactions and incorporating these interactions into more sustainable agricultural practices.

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Attribution

Victoria Meakem conducted all field and laboratory experiments, performed laboratory procedures, produced all figures and analyses, and wrote the manuscripts. Susan Whitehead contributed expertise on project design, methods of phenolic extraction and analysis, and manuscript revisions. Gustavo Teixeira and Grace Florjancic assisted with sample collection and phenolic extractions. Michael Wisniewski and Erik Burchard provided training for sequencing and analysis of microbiome data.

INTRODUCTION

Plants are constantly interacting with the biotic organisms that surround, and even reside within, their tissues. These interactions are highly diverse and may range from defending against antagonists such as herbivores and pathogens to attracting mutualists such as pollinators, seed dispersers, and beneficial microbial taxa. Furthermore, these interactions may profoundly impact plant traits, particularly in the case of closely associated microbial taxa in plant tissues, which may greatly influence the overall health and growth of their host (Berendsen et al. 2012). It has been suggested that an improved understanding of plant-microbe interactions will revolutionize agricultural methods, similar to the way enhanced understanding of the human gut microbiome has altered human health strategies (Mendes and Raaijmakers 2015; Busby et al. 2017). Thus, there is a growing need to understand how plants may respond to and shape the biotic communities around and within them.

An important way in which plants mediate interactions is through the production of a diverse array of phytochemical compounds. When a plant is challenged by an antagonistic interaction, it may respond with an upregulation of phytochemical defenses including a diverse mixture of deterrent compounds accumulated within plant tissues (Bennett and Wallsgrove 1994) as well as the release of predator-attracting volatile compounds (Kessler and Baldwin 2001). These induced responses may be systemic, occurring throughout all parts of the plant (Karban and Baldwin 2007), and have also been shown to prime plant systems to react more efficiently to future attack (Dangl and Jones 2001; Chisholm et al. 2006). However, much remains unknown regarding the adaptive function and nature of induced defenses, including the level of specificity of these defenses against target antagonists and the potential tradeoffs that may occur during multi-species attack, as well as the extent to which these defenses occur across plant taxa and tissue types (Stamp 2003; Kempel et al. 2011; Agrawal 2000; Kaplan et al. 2008; Rostás et al. 2003). Thus, there is a need for improved understanding of induced defenses; particularly in the context of agricultural systems, as these defenses may offer a new direction for improving sustainable growing practices.

Manipulating plant immune responses and the production of phytochemicals that shape plant-insect-microbe interactions offer intriguing possibilities for improving sustainable agriculture methods, particularly disease control. Historically, pest and pathogen management strategies have relied on pesticide use, which, although initially effective, often results in

subsequent rises in pest resistance (Palumbi 2001) and in ecological damage (Beketov et al. 2013; Goulson et al. 2015). In contrast to pesticides, which are often based on a single mode of action, natural plant defenses may offer increased resilience to pest and pathogen counter-adaptations due to their multifaceted nature. Furthermore, many phytochemicals that contribute to plant resistance have also historically been of interest for their health-promoting and culinary properties (Dai and Mumper 2010; Tomas-Barberan and Espin 2001). Thus, enhancing the phytochemical concentrations or diversity in crop tissues could theoretically produce agricultural products that are simultaneously better-defended and of higher quality for human consumers. Crop phytochemistry can be manipulated by novel agricultural methods, such as exogenous hormone application (Gozzo 2003), genetic engineering (Birkett and Pickett 2014), or potentially by microbial inoculations. For instance, some microbial symbionts have been shown to induce a plant defensive response (Pineda et al. 2010) as well as synthesize their own defensive metabolites (Ludwig-Müller 2015), some of which have already been utilized to prevent fungal rots in post-harvest fruit storage (Jiang 1997). In short, interaction-driven phytochemical changes in plant traits have important implications for improving crop yield and quality, and may represent an exciting avenue for the development of novel crop management strategies.

Plant phytochemical responses to biotic interactions may occur across all plant tissues, including the roots, leaves, flowers, and fruits, but fruits represent a uniquely fascinating region for examining interaction-driven phytochemistry, as they must simultaneously attract dispersers while defending against antagonists (Whitehead and Bowers 2014), and are often the commodity produced for human consumption in crop species. Thus, fruits represent a hotspot of interactions among human consumers, pests and pathogens, and seed dispersers. However, fruits have not been as well-studied as other plant organs. Thus far, most work linking biotic interactions and phytochemistry has focused on interactions occurring in the roots, where there is extensive work on plant-microbial relationships in the rhizosphere, or in the leaves, where plant induced defensive responses to herbivory have also been an area of focus. In contrast, the role of induced defenses in fruits has been largely overlooked; in fact, it was once expected that reproductive tissues such as fruits were most likely to be defended primarily by constitutive defenses, given their high value (McKey 1974; Rhoades and Cates 1976; McCall and Karban 2006). However, it has since been observed that induced defenses can occur in fruits (McCall and Karban 2006), although most work has focused on the application of defense-inducing elicitors as a pest control

strategy (Ruiz-García and Gómez-Plaza 2013), while natural fruit induced responses to different types of biotic interactions remain poorly understood. Additionally, very little is known regarding the community structure of the endophytic communities residing within fruit tissues and their impact on plant traits (Droby and Wisniewski 2018). Thus, the relationship between the highly diverse interactions occurring within fruits and their induced phytochemical responses remains an open and important area of study.

My research uses fruits from apples, an economically important and ecologically complex perennial crop, as a model system to assess two overarching objectives related to biotic interactions and phytochemistry: 1) to examine how communities of distinct biotic interactions among plants, insects, and microbes impact apple fruit chemistry (Chapter 1), and 2) to examine the impact of exogenous hormone application on induced fruit chemical responses and resistance against pests and pathogens in apples (Chapter 2). In my first chapter, I used pesticide and microbial inoculation treatments in an experimental orchard to create distinct biological communities by removing or adding specific community components. I first characterized the biotic communities created by these treatments through fruit damage evaluations to assess insect feeding and pathogen occurrence, and through sequencing of the fruit fungal endophyte communities present in different tissue types (seed, skin, and pulp). I then quantified the phenolic concentrations and diversity in these tissues as well as identified individual compounds that were potentially upregulated in response to these distinct biotic fruit communities. The potential for temporal or genotypic variability of these induced responses was assessed by comparing effects on two cultivars across two sampling periods. Finally, I assessed the relationship between fungal endophyte diversity and phenolic chemical diversity in individual fruits. In my second chapter, I applied treatments to apple fruits of plant hormones known to induce plant defensive responses in other systems. These were applied both individually and simultaneously in order to investigate potential antagonistic crosstalk effects across pathways. I then used bioassays with insect pests and fungal fruit pathogens, also conducted individually and simultaneously, to assess efficacy of these hormone treatments in mitigating damage from multi-species attack. In all, these chapters will examine the relationship between fruit chemistry and biotic interactions with the goal of improving understanding of plant-microbe-insect interactions and the potential for incorporating these interactions into more sustainable agricultural practices.

CHAPTER 1: The effect of distinct biotic communities on induced phenolic chemistry of apple fruits

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Abstract

Plants synthesize a remarkably diverse array of phytochemicals, which may be produced as an induced response to a wide range of biotic interactions. Plants interact simultaneously with organisms such as herbivores, pathogens, and commensal microbes. However, how plants respond chemically to these diverse biotic interactions, when occurring both in unison and independently, remains unclear, despite the potential importance of these responses for agricultural applications such as pest management. To examine this relationship between differing types of interactions and induced phytochemical responses, we created distinct biotic communities on apple fruits by removing or adding community components through the application of five pesticide and holistic organic treatments: a combined pesticide treatment designed to minimize both microbial and insect interactions (IM: insecticide, fungicide, and bactericide), an antimicrobial treatment designed to decrease microbial interactions only (M: fungicide and bactericide), an insecticide treatment designed to decrease insect interactions only (I: insecticide), an unsprayed treatment designed to expose fruits to both insect and microbial interactions (U), and a holistic organic treatment containing organic amendments and microbial inoculants designed to foster a potentially beneficial microbial community (O). First, we characterized the biotic communities established by these treatments through visual fruit damage evaluations and sequencing of the associated fungal endophyte communities in fruit skin, pulp, and seed tissues. We then assessed the effects of these biotic communities on phenolic concentrations and diversity in fruit tissues, and also examined how these relationships varied across two sampling periods and two cultivars. Finally, we assessed the relationship between fungal endophyte diversity and phenolic diversity within individual fruits. We found that low insect but high fungal damage levels obscured fine-scale distinctions between treatment groups, and instead generally created two broad categories: antimicrobial treated (IM and M) and non-antimicrobial treated (I, U, and O) fruits. Fungal endophyte communities were strongly affected by the presence of sooty blotch and flyspeck (SBFS), as surfaces of U and O-treated fruits were covered by the SBFS complex, and these treatments contained lower skin endophyte diversity than the IM treatment. Treatment effects on phenolic compounds varied by sampling season,

tissue type, and cultivar, such that ultimately only late-season pulp tissues displayed an induced response to fungal damage, as these tissues contained higher total phenolic concentrations and richness in the non-antimicrobial treatments that sustained higher levels of fungal damage (I, U, and O). However, there were some compounds consistently upregulated in response to fungal damage across sampling season and tissue type, including an unknown benzoic acid and, to some extent, chlorogenic acid. Furthermore, we found that fruit phenolic diversity increased with fungal endophyte diversity, as predicted, in pulp tissues. Overall, these results suggest that the strength of induced responses in fruits is context-dependent, and may vary by season, tissue type, or cultivar, and that detection of induced defenses to biotic interactions may also be confounded by abiotic conditions; however, pulp tissues may represent an important region for induced defenses, and fungal endophytes may play a role in shaping fruit pulp phenolic composition and may lead to increased phenolic diversity. Despite this complexity, linking plant-insect-microbe interactions and phytochemical responses represents an important area of investigation for understanding plant microbial community structure and plant defensive responses, both of which have profound implications for improving sustainable agricultural practices.

Introduction

Plant interactions with diverse organisms, including herbivores, pathogens, seed dispersers, pollinators, and microbial partners, are mediated through the production of phytochemicals. These chemicals can be present constitutively throughout plant tissues, or can be produced in an induced response to biotic interactions or plant damage (Karban and Baldwin 2007). The activation of these induced defenses can be rapid, as plants are able to identify a biotic interaction, such as leaf damage from a feeding herbivore, and produce chemical defenses directly against that antagonist (Karban and Baldwin 2007). Furthermore, once damaged, the plant may be primed for future attack, and is quickly able to mobilize defenses (Kim and Felton 2013; Conrath 2009), often in a systemic response across all parts of the plant (Karban and Baldwin 2007). Consequently, the biotic milieu with which plants interact may alter the phytochemical composition, concentrations, and diversity present in plant tissues, although the exact nature of the relationship between induced phytochemistry and biotic interactions remains unclear (Agrawal 2011; Wetzel and Whitehead 2020). Furthermore, these biotic interactions may also profoundly alter plant traits, including economically important agricultural traits that

influence crop yield or quality. Thus, incorporation of beneficial interactions into management strategies may lead to more sustainable and productive agricultural practices. For example, fostering a beneficial soil microbial community has been shown to increase yields and provide defense against biotic antagonists (Zamioudis and Pieterse 2012; Lakshmanan et al. 2014; Bender et al. 2016) and tri-trophic interactions can be manipulated as a biocontrol technique by enhancing the phytochemical attraction of parasitoids and predators that aid in plant defense (Lewis and Papavizas 1991; Agrawal 2011; Cardinale et al. 2003). In short, examining interaction-driven chemical changes in plant tissues will not only improve understanding of how plants respond to varying types of biotic pressures, but also represents an exciting avenue for the development of novel crop management strategies.

Plants may respond to antagonistic biotic interactions with an induced chemical response, which is suggested to be a cost-saving adaptation that allows plants to activate defenses only when necessary, avoiding the expense of continuous production (Karban and Baldwin 2007). These defenses may also be targeted to a specific attacker; for example, plants are able to recognize elicitors in the saliva of a caterpillar and produce defensive responses tailored to that antagonist (Alborn et al. 1997; Bonaventure 2014), as plants are known to activate distinct defense pathways in response to different types of attackers. Chewing Lepidoptera, for instance, activate a different defense pathway than biotrophic fungal pathogens (Glazebrook 2005). It may be that this defensive response relies on the production of a single dominant defense compound (Shen et al. 2018; Nuessly et al. 2007), but it is more common that a blend of chemical compounds is used defensively by the plant, with possible synergistic effects among compounds (Richards et al. 2016). Indeed, a plant's need to respond to a multitude of biotic interactions (i.e., Interaction Diversity Hypothesis) and its production of these synergistic compound mixtures (i.e., Synergy Hypothesis) may explain why plants contain such a high level of chemical diversity (Wetzel and Whitehead 2020; Richards et al. 2016; Berenbaum and Zangerl 1996). However, much is still unknown regarding the role of biotic interactions in driving phytochemical diversity, as well as the extent to which induced responses may be altered when a plant confronts a single antagonist in contrast to multi-species attack.

The hormone pathways that defend against certain classes of insects and fungal pathogens are distinct, and may result in the production of distinct downstream products (Schweiger et al. 2014), but there may also be overlap in metabolite production (De Vos et al.

2005), making the level of specificity of plant induced defenses difficult to determine. Furthermore, the extent to which these pathways produce distinct downstream products within a single class of phytochemicals is unclear. Phenolics, one of the largest phytochemical compound classes, are constitutively present in plant tissues, but may also be produced in an induced response to both insect and fungal damage, and have been shown to defend against a variety of pests and pathogens across crop species (Lattanzio et al. 2006). Some individual phenolic compounds, such as chlorogenic acid, provide defense against both types of antagonists; this may be due to compound-driven physiological changes within plant tissues that provide broad protection across antagonist types, such as the function of phenolics in strengthening cell walls (Santiago et al. 2005), which may defend against both chewing herbivores and tissue-invading pathogens (Lattanzio et al. 2006). The tailoring of plant defensive responses to attackers through the activation of distinct hormone pathways, however, indicates that there are likely different compounds upregulated in response to specific attackers as well. Adding to this complexity is the possible synergisms that may occur between compounds, such as an observed increased effectiveness in inhibiting fungal damage during simultaneous application of chlorogenic acid and phloridzin compared to individual applications (Lattanzio et al. 2006). Thus, there is a need to improve understanding of how different combinations of biotic interactions, specifically, fungal-driven, insect-driven, and combined fungal-insect-driven interactions, may alter the composition and diversity of downstream metabolic products, particularly within a single class of phytochemicals, such as phenolics.

In addition to responding to interactions with external insect herbivores and fungal pathogens, plants may also respond chemically to an internal associated microbial community. However, very little is known about the relationship between phytochemical production and plant microbiome community structure. Most existing research has focused on the interplay between root exudates and soil microbial communities in the rhizosphere (Sasse et al. 2018). Plant exudates may attract microbial mutualists (Rasmann and Turlings 2016) that colonize the roots and induce plant defenses in both belowground and aboveground tissues, which has been shown to reduce herbivory damage (Pineda et al. 2010). Furthermore, defensive benefits conferred by microbial mutualists have also been observed in the phyllosphere, where leaf endophytes have been shown to alter plant chemistry and reduce damage from leaf-cutter ants (Estrada et al. 2013). In addition to inducing host plant defenses and altering phytochemical

composition and concentrations, associated microbial taxa also produce their own defensive metabolites, which function to interact with both the plant host and other microorganisms, further shaping the interactions that take place within plant tissues (Kusari et al. 2012; Lugtenberg et al. 2017). Indeed, fungal endophytes have been observed to be prolific producers of phytochemicals (Schulz et al. 2002; Ludwig-Müller 2015) including phenolics (Subban et al. 2013). However, the relationship between phytochemistry and microbiome communities remains largely unexplored, despite its important implications for agricultural management practices. Similar to how human health strategies have been shaped by improved understanding of the human gut microbiome, it has been proposed that sustainable agriculture practices and plant health will also be dramatically improved by incorporation of benefits derived from the plant microbiome (Busby et al. 2017; Mendes and Raaijmakers 2015).

Although insects, fungal pathogens, and the plant-associated microbiome have all been shown to alter plant chemistry, much is still unknown about how simultaneous exposure to these different classes of biotic interactions may alter plant chemical defenses. It is possible that plants may prioritize the defense against one type of antagonist over another; indeed, crosstalk that exists among plant hormone pathways has been viewed as an adaptation that allows plants to inhibit one type of defense in favor of another (Glazebrook 2005; Thaler et al. 2012). However, it is also possible that, once induced, the overall heightened levels of chemical defenses in plant tissues may allow for protection across antagonist type; indeed, it has been suggested that specificity in antagonist recognition does not necessarily result in the production of specific downstream metabolites (Paul et al. 2000). However, there have been examples of plants producing specific compounds in response to specific antagonists (Stout et al. 1997). Thus, if plants do produce distinct metabolites in response to specific interactions, it is likely that the more diverse biotic pressures a plant experiences, the more varied and heightened its chemical responses will be, indicating that as the diversity of different types of biotic interactions with the plant increases, the diversity of phytochemicals it produces may increase as well. For instance, a plant exposed to both fungal and insect damage may contain a more highly diverse chemical profile than a plant exposed solely to insect damage. Thus, we generally predict a positive relationship between biotic diversity and chemical diversity, resulting from the various tailored responses that plants employ against different types of antagonists.

Fruit communities provide a particularly fascinating and complex setting for examining interaction-driven phytochemistry. Optimal defense theory suggests that these organs should be highly protected due to their importance and high cost of production (McKey 1974; Rhoades and Cates 1976; McCall and Karban 2006); however, their primary ecological function is to be consumed by seed dispersers, suggesting a selective pressure against defenses that deter dispersers (Whitehead and Bowers 2013b). Plants may resolve these conflicting pressures through temporal variation in defenses, decreasing fruit phytochemical content during ripening (Whitehead and Bowers 2013a; Tsahar et al. 2002). Thus, temporal variation represents an important consideration when examining fruit defenses. Furthermore, the role of induced defenses in fruits has not been well-studied; although induced defenses have been observed to exist in fruits (McCall and Karban 2006), very little work has been done examining fruit phytochemical responses to distinct biotic interactions within different fruit tissues and across plant taxa. Given the conflicting selective pressures on fruits, however, the flexibility offered by induced defenses of dynamically increasing or decreasing phytochemical content in response to distinct interactions suggests the potential for these defenses to play a significant role in fruit tissues.

Similar to induced defenses, endophyte communities have also been less well-studied in fruits than in roots or leaves. Those few studies that have characterized the fruit microbiome have focused on comparing conventional, organic, and postharvest treatments in agricultural systems (Droby and Wisniewski 2018). Most of these treatments have been shown to alter microbial community structure; for instance, organic and conventionally-managed apples were found to contain distinct fungal (Abdelfattah et al. 2016) and bacterial (Wassermann et al. 2019b) communities. Furthermore, there is a growing interest in developing strategies that foster a beneficial fruit microbial community in which key taxa maintain a community structure resistant to invasions from destructive pathogens, either by outcompeting these antagonists for resources, synthesizing defensive metabolites, or inducing host plant defenses (Wassermann et al. 2019a; Droby et al. 2016). However, to our knowledge, there have been no studies linking the fruit microbiome and induced host defenses, despite its importance for understanding potential mechanisms behind these beneficial community dynamics. Furthermore, there is a need to understand the variation of the fruit microbiome in response to genetic or environmental differences among host plants (Busby et al. 2017; Droby et al. 2016), as well as the spatial

variability of these communities within the fruit itself. Apple fruits were found to contain distinct fungal communities across the center, calyx end, and stem end regions of the skin surface (Abdelfattah et al. 2016), and to contain distinct bacterial endophyte communities among skin, pulp, seed, calyx, and stem tissues (Wassermann et al. 2019b). These tissue-specific patterns are likely driven by the spatial variability of distinct biotic and abiotic factors; for instance, skin tissues may be exposed to environmental stressors such as UV irradiation or desiccation, as well as a high diversity of biotic organisms interacting with the fruit. Pulp tissues may be less exposed to the external environment, but may still experience biotic intrusions such as tunneling larvae and interior-extending rots. Finally, seed tissues, which are protected by physical barriers, may contain a distinct microbial community (Nelson 2018). Thus, there is a need for studies that characterize the link between fruit induced chemical responses and the diverse biotic interactions occurring within skin, pulp, and seed tissues, and that also address the effects of temporal, environmental, and genetic variation of the host plant.

To improve understanding of the impact of distinct communities of biotic interactions and phytochemical diversity, we applied five treatment regimes to an apple orchard throughout the growing season: 1) a combined pesticide treatment designed to decrease both microbial and insect interactions (IM: insecticide, fungicide, and bactericide), 2) an antimicrobial treatment designed to decrease microbial interactions only (M: fungicide and bactericide), 3) an insecticide treatment designed to decrease insect interactions only (I: insecticide), 4) an unsprayed treatment designed to expose fruits to both insect and microbial interactions (U), and 5) a holistic organic treatment including organic amendments and microbial inoculants designed to foster a beneficial microbial community (O). We then used these treatment-established communities to evaluate three specific objectives: 1) to characterize the distinct communities of biotic interactions created by these treatments by evaluating A) visible fruit damage levels from major pests and pathogens and B) changes in the fungal endophyte communities associated with apple fruit tissues, 2) to assess the effects of these communities on fruit chemistry by quantifying the concentration and diversity of phenolic compounds across two sampling periods, representing unripe (mid-season) and ripe (late-season) fruit, and across two cultivars (‘York’ and ‘Golden Delicious’), and 3) to examine the relationship between fungal endophyte diversity and phenolic chemical diversity within individual fruits. We expected that these treatments would establish distinct biotic communities resulting in different profiles of fruit phenolic chemistry due to the induction of

tailored defensive responses, and that these patterns may vary across tissue type, sampling season, and cultivar due to the potential for environmental factors to modulate these interactions. Furthermore, we predicted that pesticide-treated fruits would contain a lower diversity of fungal endophytes and phenolic compounds than unsprayed and holistic organic-treated fruits, and that, overall, fruit phenolic diversity would increase with fungal endophyte diversity, suggesting that distinct biotic communities produce specific phytochemical responses, such that fruit communities with increased biotic diversity contain a higher phytochemical diversity as well.

Methods

Study Site

The project took place at the Virginia Polytechnic Institute and State University's (Virginia Tech) Kentland Farm, which contains a 16-year old apple orchard composed of 'York' (Y) and 'Golden Delicious' (GD) cultivars grafted on M.26 rootstock. The orchard has historically been managed with conventional spray programs that include the application of fungicides and insecticides approximately every two weeks throughout the growing season and antibiotics (streptomycin) at bloom to prevent fire blight infection. The ground is managed with conventional herbicides.

Treatments

During the summer of 2018, the following five treatment regimes were applied to trees with an airblast sprayer every two weeks from April to September: 1) combined insecticide, fungicide and bactericide treatments (IM), 2) fungicide and bactericide treatments (M), 3) insecticide treatments (I), 4) no treatment (U, unsprayed), and 5) a treatment designed by grower Michael Philips to stimulate a beneficial microbial community (O, holistic organic; Philips 2012). This holistic organic treatment contained organic amendments and effective microbes within the genus *Lactobacillus* to reduce damage from fungal pathogens, as well as neem oil, which contains the compound azadirachtin, a natural insecticide (Mordue and Blackwell 1993). In all, this treatment was designed to promote the dominance of beneficial microorganisms while minimizing invasions from antagonistic pathogens and limiting insect damage through natural defense compounds. Additional details regarding treatment applications can be found in Table 1.

Table 1. Treatments applied to trees to create distinct communities of biotic interactions.

Treatment	Predicted Biotic Community	Treatment Details
Insecticide + Antimicrobials (IM)	Low levels of insect and microbial interactions, including fungal pathogens	One treatment applied every two weeks, alternating between two regimes, each with one insecticide, one fungicide, and one bactericide: 1) Lorsban + captan + streptomycin 2) Imidan + Pristine + oxytetracycline
Antimicrobials (M)	Insect-driven interactions	One treatment applied every two weeks, alternating between two regimes, each with one fungicide (captan, mancozeb) and one bactericide (streptomycin + oxytetracycline)
Insecticide (I)	Microbial-driven interactions, including fungal pathogens	One treatment applied every two weeks, alternating between two chemicals: lorsban and imidan
Unsprayed (U)	Interactions with insects and microbial taxa, including fungal pathogens	No treatment applied
Holistic Organic (O)	Interactions with insects and microbes, including fungal pathogens, but potentially mitigated by the presence of a beneficial microbial community and natural insecticides	One treatment applied every two weeks containing a mix of probiotics and plant defense stimulators as recommended by Michael Phillips. Recipe for 20 trees: 1) 1 2/3 cup (13 oz) pure neem oil 2) 5 tsp soap emulsifier (any biodegradable soap) 3) 6 ¼ cup liquid fish 4) 3 ¾ cup effective microbes (EM-1 Microbial Inoculant, TeraGanix, Alto, TX, USA) 5) 2 ½ cups blackstrap molasses 6) 1 ½ cups liquid kelp 7) 20 gallons water.

Treatments were applied to randomized blocks of three to four trees, for a total of 13-15 trees in each treatment group across both cultivars. A row of buffer trees that received no treatment was established between each row of treated trees, and a buffer tree was also designated between each treatment block and at the end of each row in order to reduce potential cross-contamination of sprays between treatment blocks.

Sample Collection

Fruit sample collection took place twice over the growing season. First, mid-season sampling of unripe fruits occurred during the weeks of June 26 - July 13, 2018. At this time, trees were also thinned by hand so that only one or two fruits remained in each cluster. From each tree, three apples were harvested, one for immediate analysis of phenolic chemistry while two were preserved for future work, and an additional 20 fruits were harvested for pest and pathogen damage evaluations. All harvested apples were selected at random, regardless of damage extent or size, but those fruits that were clearly stunted or aborted were excluded, and the three fruits selected for phenolic analysis were collected from three different regions of the tree. The three fruits harvested for quantification of phenolic content were separated into skin, pulp, and seed tissues using a knife to collect the skin tissue and a cork borer to collect the pulp tissue. Tissue samples were placed into 2 mL microcentrifuge tubes, immediately frozen in liquid nitrogen, and stored at -80℃. For the 20 fruits selected for damage evaluations, damage was marked as present or absent for common pests and pathogens, including plum curculio (*Conotrachelus nenuphar*) and cedar-apple rust (*Gymnosporangium juniperi-virginianae*), as well as sooty blotch and flyspeck, which are caused by a complex of fungi that may be composed of over 100 species, belonging primarily to the class Dothideomycetes and the order Capnodiales (Gleason et al. 2019). All other damage present was recorded as "Other." A rating scale was used to evaluate the level of russeting, a brown, rough texture that appears on fruit surfaces, such that 1 = <5% coverage, 2 = 5-25% coverage, 3 = 25-50% coverage, 4 = 50-75% coverage, and 5 = >75% coverage.

Sampling was carried out again in the late-season after fruit ripening between October 3-19, 2018. Fruits were harvested following the same procedures used in mid-season sampling, in which three fruits were selected for phenolic analysis and 20 fruits were harvested for damage evaluations. However, due to the presence of sooty blotch and flyspeck on almost all fruits at varying levels, a rating scale was added for these pathogens, where 0 = no infection, 1 = <5% coverage, 2 = 5-25% coverage, 3 = 25-50% coverage, 4 = 50-75% coverage, and 5 = >75% coverage. Furthermore, the three apples harvested for phenolic content were also sampled for the microbial endophyte and epiphyte community. Thus, each fruit was collected with gloves that were sterilized with dilute bleach and ethanol, placed in a sterile bag (Whirl-Pak®, Nasco, Fort Atkinson, WI) and brought back to the lab. To sample the epiphyte community, we swabbed

fruits in a straight line along the widest part of the apple on a UV-sterilized Heraguard ECO Clean Bench surface (BD BBL Culture Swab, single tip; Thomas Scientific Catalog #C001Y57). Swabs were then stored at -80℃. To sample the endophyte community, apples were then washed in distilled water to remove loose debris before surface sterilization under the Clean Bench hood by submersion in the following solutions: 95% ethanol in sterile water for 30 seconds, 0.7% hypochlorite (bleach) in sterile water for 2 minutes, 70% ethanol for 30 seconds, and sterile water for ~ 5 seconds. Using a sterilized knife, the apple skin was peeled around the same line where the swab was collected. The peel was divided into 4 sections, and then each section was cut in half, with one half placed in a sterile tube for microbiome analysis and the other half placed in a non-sterile tube for phenolic analysis, so that each region of the peel would be represented in the sample. The apple was then cut into quarters, and the pulp samples were collected from each of the four exposed quarters using a cork borer, ensuring no peels were collected with the pulp. As with the skin samples, each pulp core was cut in half so that half went into the sterile tube for microbiome analysis and half went into a non-sterile tube for phenolic analysis. Seeds were collected in sterile tubes for microbiome analysis only. All tools used in tissue sample collection were re-sterilized between each fruit. Swab samples of epiphyte communities were preserved for future work, and only endophyte communities were assessed in this study. At the same time as swab and tissue collection for phenolic and microbiome analyses, fruit quality metrics such as size, firmness, fruit maturity, sugar content, and water content were also collected, and are available in a published dataset (Teixeira et al. 2020).

Phenolic Extractions

After storage at -80℃, fruit tissue samples were lyophilized, ground to powder, and extracted in 600 µL of a 70% methanol and 2% formic acid solution using trans-cinnamic acid (17 µL 1000 ppm solution) as an internal standard. The extraction involved placing tubes on a vortex mixer for 30 min at 1500 rpm, followed by centrifugation for 10 min at 10,000 rcf. The supernatant was then transferred, and two more rounds of extractions were performed on the same plant material for a total of three extractions. The combined extracts were then filtered through a 0.22 µm centrifuge tube filter (Costar Spin-X, Fisher Scientific).

Analysis of phenolic content was carried out using an Agilent 1100 high-performance liquid chromatograph (HPLC) equipped with a diode array detector (Agilent Technologies, Palo

Alto, CA, USA) and an Inertsil ODS-3 column (5.0 μm particle size, 4.6 × 250 mm, GL Sciences Inc., Tokyo, Japan) preceded by an Inertsil ODS-3 guard column (5.0 μm, 4.0 × 10 mm). We used instrument parameters and solvents following procedures of Whitehead and Poveda (2019); briefly, two solvents were used in the gradient system for separation: Solvent A (10% formic acid in water) and Solvent B (10% formic acid and 1.36% water in acetonitrile). The flow rate was set to 1.0 ml min^{-1} with an injection volume of 5 μL. The gradient was as follows: 95% A (0 min), 85% A (25 min), 78% A (42 min), 58% A (49 min), 0% A (55 min), 0% A (60 min), 95% A (62 min). Post-run time was 10 min. Simultaneous monitoring was performed at 280, 320, 365 and 525 nm.

When possible, peaks were identified by comparison of retention time and UV spectra with 22 known standards: catechin, epicatechin, procyanidin B1, procyanidin B2, phloridzin, gallic acid, syringic acid, chlorogenic acid, gentisic acid, caffeic acid, p-coumaric acid, ferulic acid, quercetin, hyperin, isoquercitrin, quercitrin, rutin, reynoutrin and avicularin (obtained from Extrasynthese, Genay Cedex, France; Sigma-Aldrich, St. Louis, MO, USA; and AApin Chemicals, Abingdon, Oxon, UK). Calibration curves from the known standards were used to determine the concentrations of individual compounds based on peak area. If no standards were available for a compound, it was classified by compound class as either dihydrochalcone, hydroxycinnamic acid, benzoic acid, flavonol, flavan-3-ol or anthocyanin based on UV spectra, and concentrations were estimated based on internal standard equivalents. Phenolic concentrations were then calculated as micrograms of phenolics per gram of dry weight (μg g^{-1} DW).

Fungal Endophyte Characterization

A subset of 48 samples were selected for sequencing of the fungal endophyte communities, representing six 'York' trees in each of the O, U, and IM treatment groups for a total of 18 trees. The skin, pulp, and seed samples were analyzed for each fruit, although seed samples were excluded from two trees in each treatment. Thus, there were six skin and pulp samples and four seed samples per treatment group. After storage at -80℃, fruit tissue samples were lyophilized, ground to powder in liquid nitrogen, and extracted using the Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Valencia, CA, USA, Cat No./ID: 12855-100). Each sample of extracted DNA was assessed for fungal endophyte communities using the ITS3 and ITS4 primers (Toju et al. 2012)

to target the ITS2 region of fungal rDNA. In order to minimize host apple DNA, a blocking primer was designed specifically for apples with the following sequence: (ATTGATATGCTTAAATTCAGCGGGTAACCCCGCCTGACCTGGGGTCGCGTT/3SpC3/). Amplification and purification was carried out following the Illumina-supplied guidelines for 16S metagenomic sequencing library preparation (Illumina 2013; Illumina, San Diego, CA, USA). Briefly, the reactions for amplicon PCR contained a total volume of 25 µL with the following components: 2.5 µL DNA, 1 µL of each primer (10 µM), 1 µL blocking primer, 12.5 µL KAPA HiFi HotStart ReadyMix (2x) (Roche Sequencing, Pleasanton, CA, USA Cat KK2602), and 7 µL sterile water. A T100 thermal cycler (Bio-Rad) was then used for thermal cycling with the following reactions times: 3 minutes at 95 ℃, followed by 25 cycles of: 30 seconds at 95℃, 30 seconds at 55 ℃, 30 seconds at 72 ℃, 5 minutes at 72 ℃, and a hold at 40 ℃. PCR products were then purified using AmPure XP Magnetic Beads (Beckman Coulter, Brea, CA, USA, Cat. A63881). Index PCR was then carried out to attach Illumina sequencing adaptors using the NexteraXT Index Kit (Illumina, Inc, San Diego, CA, Cat. FC-131-1001), and sequences were purified again with AmPure XP Magnetic Beads prior to library quantification using the Qubit dsDNA HS Kit (ThermoFisher Scientific, Waltham, MA, USA, Cat. Q32854). Samples were normalized and pooled, after which 10% PhiX control was added. Barcoded libraries were sequenced using the Illumina Miseq platform (Illumina, Inc, San Diego, CA).

Following sequencing, data processing was carried out using the Qiime2 v.2020.2 bioinformatics platform (Caporaso et al. 2010). After joining paired sequences and demultiplexing using standard settings for paired end sequences with quality, the DADA2 plugin was used to denoise sequence data by removing low-quality reads and chimeras (Callahan et al. 2016). The DADA2 plugin was used with standard settings (i.e., expected error value of 2, truncation of reads at quality scores less than 2, and use of "consensus" method for chimera removal), and sequences were also truncated at a length of 238 base pairs from the 3' end for forward read sequences and at 201 base pairs for reverse read sequences due to a decline in quality. Furthermore, the first 40 base pairs from the 5' end were also trimmed due to low read quality. Taxonomy was then assigned using the UNITE database, version 8.2 (Nilsson et al. 2019) using the "dynamic" representative sequence file in which taxonomic experts indicated the level at which each species should be clustered (i.e., 97 or 99%). The amplicon sequence variant (ASV) tables were then exported to the R programming environment, where samples were

rarefied at an even sampling depth of 3494 prior to analyses. Two samples with fewer sequences were excluded from analyses.

Statistical Analyses

To analyze the fruit damage evaluations assessing biotic community differences among treatments (Objective 1A), damage from dominant antagonists was first classified as fungal or insect. The proportion of undamaged, fungal-damaged, or insect-damaged fruits were then analyzed separately, with binomial counts of undamaged (vs. damaged), fungal-damaged (vs. non-fungal damaged), and insect-damaged (vs. non-insect damaged) fruits as the response variables in generalized linear mixed models (GLMMs) with a binomial distribution using the R package lme4 (Bates et al. 2020). In these models, cultivar and treatment were evaluated as fixed effects, and tree and experimental block were assessed as nested random effects. The mid and late-season sampling periods were evaluated in separate models. In some cases, due to complete separation of the data points (i.e., all fruits within a treatment group sustained damage and displayed a value of 1), we used pseudo-data to reduce model bias by randomly designating one fruit from each cultivar type as uninfected to allow for successful implementation of the model, following a procedure similar to that of (Kosmidis 2019). Rating scales of russeting, sooty blotch, and flyspeck damage, which ranged in value between 0-5, were assessed as predictor variables in linear mixed models (LMM) with cultivar and treatment as the fixed effects and tree and block as nested random effects. The mid and late-season sampling periods were evaluated in separate models. For all GLMM and LMM models, statistical support for fixed effects as predictors was assessed by likelihood ratio tests comparing nested models with single terms deleted. A trend in separation among treatment groups or cultivars was considered significant if the p-value fell below 0.05, and marginal if the p-value was less than 0.1. In cases where we detected significant effects of treatment, this was followed by Tukey HSD tests to assess pairwise comparisons among treatment groups using the multcomp package (Hothorn et al. 2020).

To evaluate the impacts of treatments on fungal endophyte communities for a subset of late-season ‘York’ samples (Objective 1B), sequence data was processed using the package phyloseq to rarefy samples at an even sampling depth of 3494 (McMurdie and Holmes 2013). We then calculated distance using the Bray-Curtis dissimilarity matrix followed by a

permutational multivariate analysis of variance test (PERMANOVA) to detect overall differences in community composition among treatments and tissue types. Post-hoc comparisons among groups were analyzed using a pairwise adonis test (Martinez 2019). To assess differences among treatment groups in the dispersion among samples (i.e. beta-diversity), we used a test for variance in multivariate homogeneity of group dispersions (function "betadisper" in the R package vegan), followed by a post-hoc Tukey HSD test. Distances in overall community similarities were visualized using a principal coordinate analysis ordination plot (PCoA). We then assessed alpha diversity, or diversity within samples, using the R package vegan (Oksanen et al. 2019), by calculating the following metrics: richness, which is defined as the number of taxa in each sample, Pileou's evenness, which describes how equally abundant these taxa are within samples, and Shannon Diversity Index (H), which incorporates both evenness and richness into its value. We analyzed the effect of treatment on alpha diversity separately for each diversity metric and tissue type using linear mixed models with treatment as the fixed effect and the experimental orchard block as the random effect, followed by a Tukey HSD test to assess pairwise comparisons among treatment groups. Finally, dominant taxa were visualized using stacked bar plots of relative abundance at the class and genus taxonomic levels. Furthermore, to identify key taxa at the class or genus level that varied among treatment groups, we used random forest classification models (Ranganathan and Borges 2010), and selected variables using the packages randomForest (Cutler and Wiener 2018) and Boruta (Kursa and Rudnicki 2018). Separate models were used for each tissue type and taxonomic level. The relative abundance of these taxa within each tissue type was then assessed using linear mixed models with treatment as the fixed effect and the experimental orchard block as the random effect, followed by a post-hoc Tukey HSD test.

To analyze the effect of each community of biotic interactions on fruit phenolic chemistry across sampling seasons and cultivars (Objective 2), we carried out the same analyses used to assess the endophyte community; specifically, PERMANOVA and dispersion analyses were conducted to compare differences in phenolic composition and variance among treatments, and Shannon Diversity Index (H), richness, and Pileou's evenness were also calculated to assess alpha diversity of phenolic compounds, for each sampling season. Treatment effects on these metrics were evaluated for each tissue type using linear mixed models with experimental block as the random effect and treatment and cultivar as the fixed effects. To compare effects of

sampling season on phenolic concentrations and diversity metrics, we used linear mixed models with season and cultivar as the fixed effects and tree as a nested random effect within the experimental block. If distributions were non-normal, values were first log-transformed. For all models, a likelihood ratio test (LRT) was used to determine whether there was an interaction between treatment and cultivar; if the interaction was significant, treatment effects were then assessed separately by cultivar. A Tukey HSD test was then conducted to assess pairwise comparisons among treatment groups. To identify individual compounds that varied among treatment groups, we used random forest classification models, as specified above. Separate models were used for each tissue type and sampling season, and cultivars were examined in both combined and separate models.

Finally, to examine the relationship between fungal endophyte diversity and phenolic chemistry (Objective 3), we used a linear regression with treatment and fungal endophyte diversity metrics (i.e, richness, evenness, or Shannon diversity) as the predictor variables, and phenolic chemistry diversity metrics (richness, evenness, or Shannon diversity) as the response variables. Pulp and skin samples were assessed separately.

All statistical analyses were performed in R v3.6.1 (R Core Team 2020), and all figures were produced using the packages ggplot2 (Wickham et al. 2019), cowplot (Wilke 2019), and gridExtra (Auguie and Antonov 2017).

Results

Fruit damage evaluations

Overall, treatments generally created different levels of apple fruit damage (Fig. 1). The antimicrobial treatments in particular mitigated damage, creating a strong pattern in which substantially higher numbers of undamaged fruits and lower numbers of fungal-damaged fruits were found in antimicrobial-treated trees (M and IM treatments) than in non-antimicrobial-treated trees (O, I, and U) for both the mid-season and late-season sampling periods, as expected (all $p < 0.0278$; Fig. 1 A-B, E-F). Furthermore, during the late-season, the IM treatment contained lower numbers of fungal-damaged fruits ($p = 0.004$) and marginally higher numbers of undamaged fruits than the M treatment ($p = 0.0795$), although this was driven primarily by the 'Golden Delicious' cultivar (Fig. 1B, F).

Although fungal damage was reduced in the antimicrobial treatments compared to other treatments, a high percentage of antimicrobial-treated fruits still sustained some type of fungal damage by the end of the growing season (Fig. 1). This was driven by the high abundance in the orchard of the sooty blotch and flyspeck pathogens (SBFS), for which a rating scale was added, revealing that the IM and M treatments sustained substantially lower SBFS damage levels compared to I, O, and U treatments (all $p < 0.001$; Fig. 2A-B). Interestingly, SBFS infection levels in the O treatment, although still higher than in M and IM, were significantly reduced compared to the U and I treatments (all $p < 0.01$; Fig. 2A-B).

In contrast to fungal damage, treatment effects on insect damage varied by sampling season. During the mid-season, fruits treated with insecticide (I and IM treatments) sustained lower or marginally lower levels of insect damage than other treatments (U, O, and F; all $p < 0.0979$), as expected. During the late-season, however, this pattern disappeared, and the only marginal effect of insecticide was higher insect damage levels in the O treatment compared to IM ($p = 0.0531$). When cultivars were assessed separately, 'Golden Delicious' fruits only varied in insect damage between O and M treatments ($p = 0.0280$), and although 'York' fruits displayed a trend of increased damage in O, M, and U treatments, as expected, it was not significant (all $p > 0.25$; Fig. 1D).

Finally, the rating scale for russeting, a distinct physical malformation observed in fruits that could not be classified as either fungal or insect damage, was substantially higher in the O treatment compared to all other treatment groups in both the mid-season and late-season sampling periods, but only for 'Golden Delicious' fruits (all $p < 0.001$; Fig. 3A-B).

Overall, the 'York' cultivar sustained higher damage levels than 'Golden Delicious'. Specifically, 'York' apples contained higher levels of late-season sooty blotch and flyspeck infection (both $p < 0.003$), as well as higher overall fungal damage in both the mid-season and late-season (both $p < 0.001$), marginally higher late-season insect damage ($p = 0.0871$), and lower numbers of undamaged fruits in both the mid-season ($p < 0.001$) and late-season ($p = 0.00258$). In contrast, 'Golden Delicious' fruits sustained higher levels of russeting than 'York' during both the mid and late-season (both $p < 0.002$).

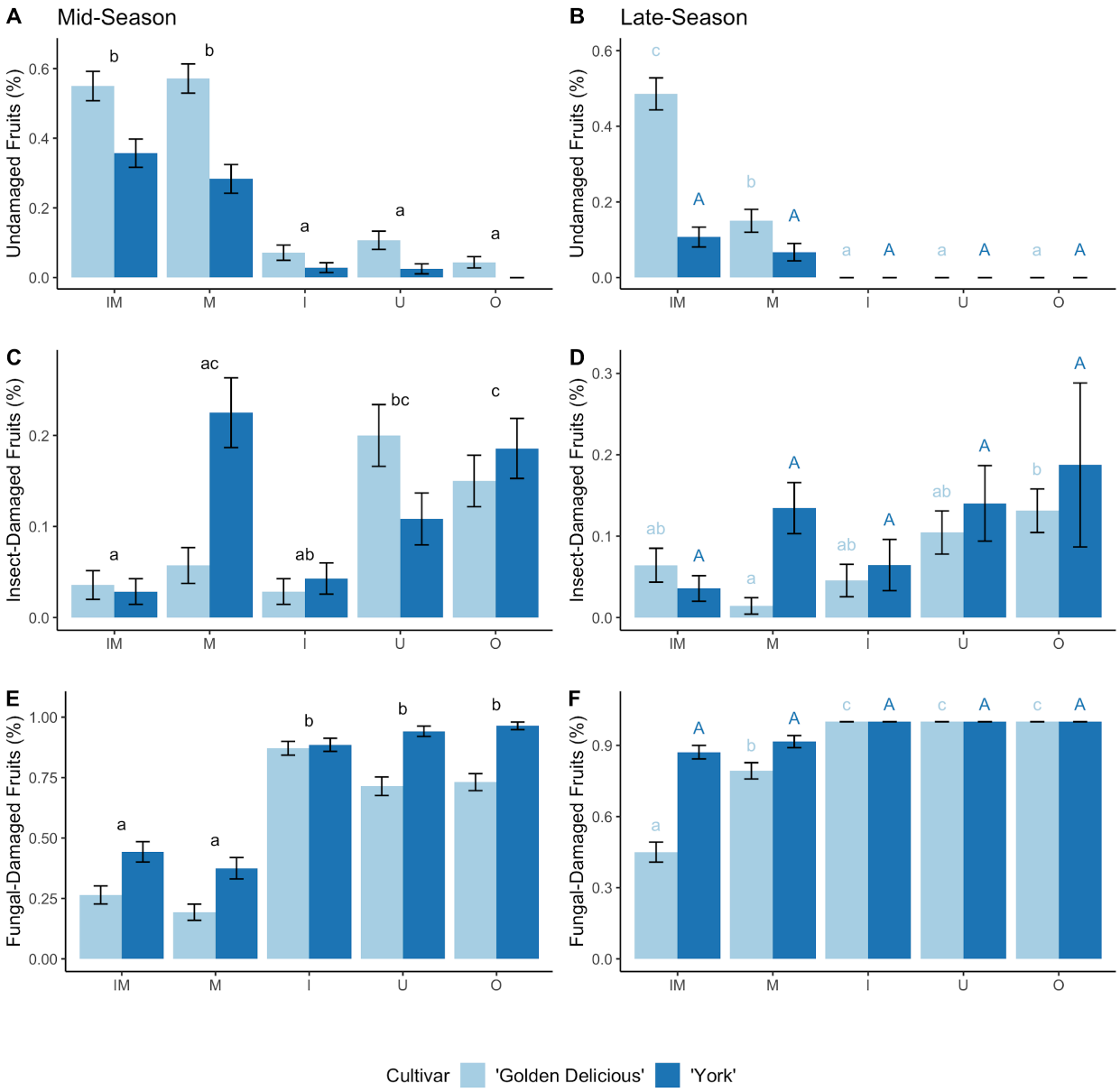

Figure 1. Percent of fruits that were undamaged, insect-damaged, or fungal-damaged during the mid-season (A, C, E) or late-season (B, D, F) in the following treatment groups: IM (insecticide + antimicrobial), M (antimicrobial), I (insecticide), U (unsprayed), and O (holistic organic). Bars indicate standard error, and letters denote significant differences among treatment groups ($p < 0.05$). When cultivars were assessed separately, letter colors correspond to cultivar color.

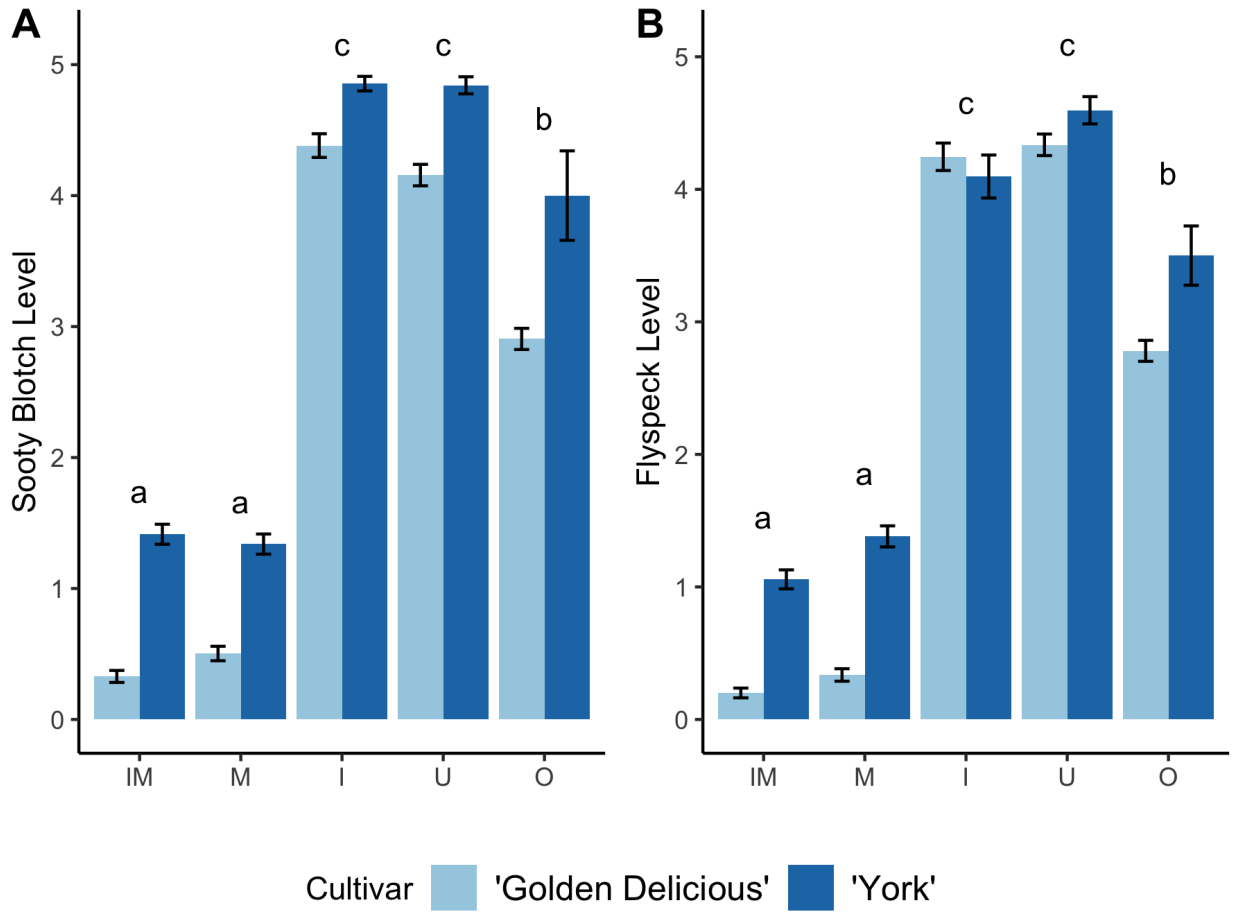

Figure 2. Rating scale levels of A) sooty blotch and B) flyspeck infection in late-season fruits across the following treatment groups: IM (insecticide + antimicrobial), M (antimicrobial), I (insecticide), U (unsprayed), and O (holistic organic). Bars indicate standard error. Letters denote significance among treatment groups ($p < 0.05$) across both cultivars.

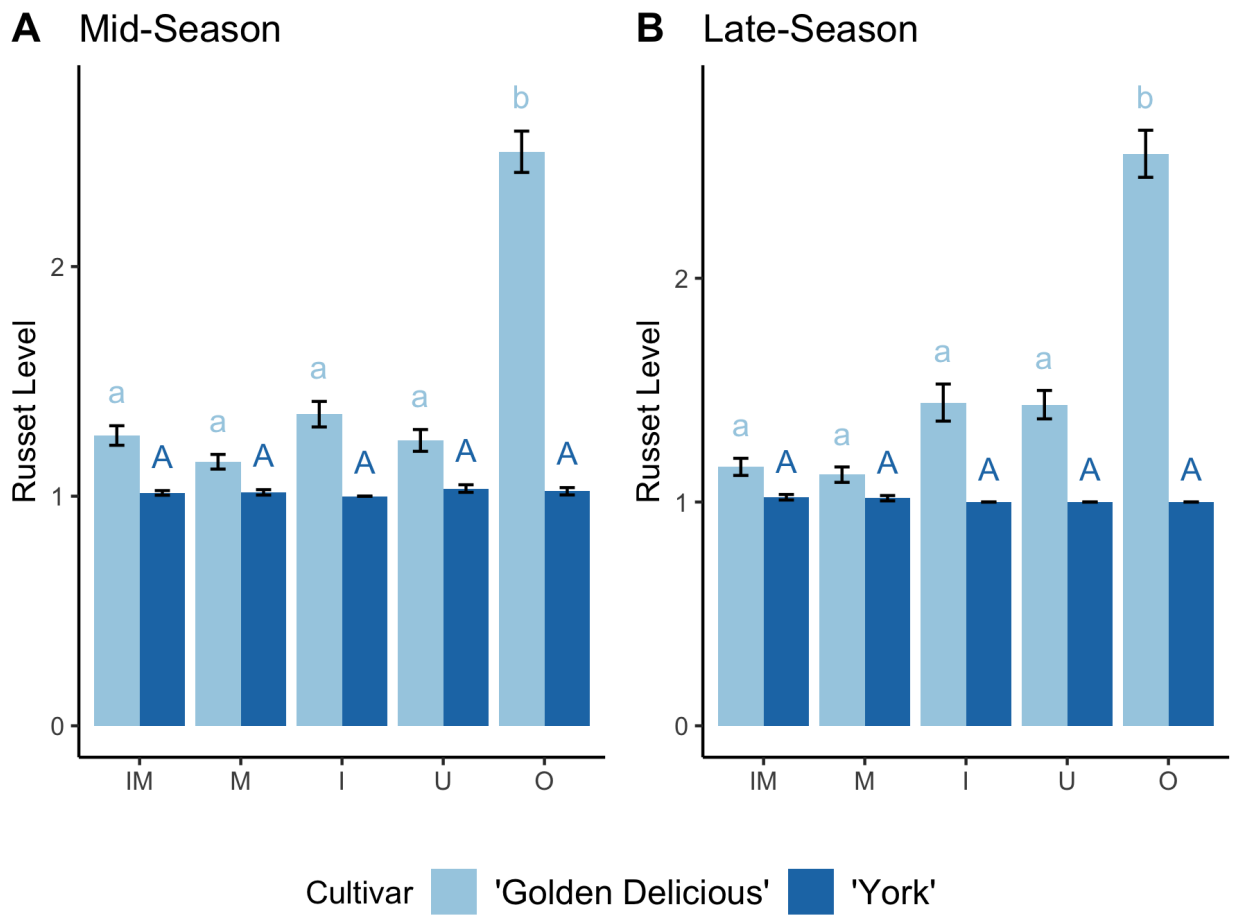

Figure 3. Rating scale levels of russeting in A) mid and B) late-season fruits after application of the following treatments: IM (insecticide + antimicrobial), M (antimicrobial), I (insecticide), U (unsprayed), and O (holistic organic). Bars indicate standard error. Letters denote significance among treatment groups ($p < 0.05$), and letter colors correspond to cultivar color.

Fungal Endophyte community composition

For the subset of fruit tissue samples analyzed for fungal endophyte communities, there were overall treatment effects on community composition (PERMANOVA; $p = 0.001$), such that pesticide-sprayed (IM), holistic organic (O) and unsprayed (U) fruits all contained distinct communities (all $p < 0.042$; Fig. 4). Community structure also varied by tissue type ($p = 0.001$), such that seed tissues harbored a distinct community from that of pulp or skin tissues (both $p = 0.003$; Fig. 4). When assessing the dispersion within each treatment group, there was an effect of tissue, as seed samples displayed lower variance than pulp ($p = 0.001$) and skin ($p = 0.006$), but there was no effect of treatment (all $p > 0.106$; Fig. 4).

While there were also some treatment effects on the alpha diversity metrics of Shannon diversity, evenness, and richness, these patterns varied across tissue types. Skin samples contained higher Shannon diversity in IM treatments compared to U ($p = 0.0206$), while O displayed a trend of intermediate diversity (Fig. 5C). This was driven primarily by the higher evenness in the IM and O treatments than U (both $p < 0.0334$, respectively), as there were no differences among treatments in skin richness (all $p > 0.503$). Within pulp tissues, there were no treatment effects on any alpha diversity metrics (all $p > 0.364$; Fig. 5), although IM evenness was marginally higher than O ($p = 0.0721$). Interestingly, treatment effects on seed richness displayed an opposite pattern to that of skin Shannon diversity, as seed samples contained higher richness in the U treatment compared to IM ($p = 0.00145$), while O was again intermediate (Fig. 5A). However, there were no differences in seed evenness or diversity among treatments (all $p > 0.592$). Overall, seed samples contained the highest fungal endophyte diversity (both $p < 0.0415$) and richness (both $p < 0.001$) of all tissue types, but there were no differences in evenness (all $p > 0.303$; Fig. 5).

There were several identifiable taxa that appeared consistently across treatments and tissues, but their relative abundance varied. At the class-level, Eurotiomycetes and Dothideomycetes dominated across tissue types, comprising 70.9% of relative abundance in skin, 60.0% in pulp, and 55.4% in seed tissues (Fig. 6), but their relative abundance varied by treatment. Unsprayed skin samples contained higher percentages of Dothideomycetes (78.9%) than both IM (34.5%; $p = 0.0105$) and O (38.8%, $p = 0.0095$), in contrast to Eurotiomycetes, which was marginally higher in IM (32.7%) than U (10.04%; $p = 0.096$). Additionally, Saccharomycetes was identified as an important class among treatment groups in the random forest model (Table 2), and constituted a higher percentage of the IM treatment (3.5%) than U (0.048%; $p = 0.007$) and, marginally, O (0.88%; $p = 0.055$). Furthermore, Sordariomycetes percentages were marginally higher in the O treatment (25.4%) than U (5.3%; $p = 0.05$) and IM (5.7%; $p = 0.099$). Pulp tissue patterns were somewhat consistent with skin, as Dothideomycetes comprised a higher relative abundance in O (55.7%, $p = 0.009$) and U (49.7%, $p = 0.04$) than in the IM (16.5%) treatment, Eurotiomycetes tended to be higher in IM (28.8%) than O (13.1%) and U (16.0%), and Sordariomycetes again tended to dominate the O treatment (O: 15.3%, U: 6.9%, IM: 10.5%; Fig. 6). Finally, seeds contained the highest percentage of unidentified taxa at the class level among all tissues (IM: 53.9%, O: 22.5%, U: 20.9%), but was also dominated by

Dothideomycetes (O: 29.3%, U: 21.9%, IM: 17.9%) and Eurotiomycetes (O: 35.7%, U: 45%, IM: 15.5%), as in other tissues. Malasseziomycetes was selected as an important class among treatments in seed samples by the random forest model (Table 2), and only appeared in the U treatment, but at low percentages (0.41%).

At the genus level, O-treated skin samples were dominated by *Colletotrichum* (24.1%), *Zygophiala* (22.4%), and *Penicillium* (13.9%; Fig. 7). While these three genera were also the most abundant in the U treatment, *Zygophiala* (70.5%) vastly outnumbered both *Penicillium* (9.7%) and *Colletotrichum* (4.1%). In contrast to the O and U treatments, the IM treatment contained low percentages of *Zygophiala* (<1%) and *Colletotrichum* (2.7%), and was instead dominated by *Penicillium* (31.6%), *Aureobasidium* (17.6%), and *Alternaria* (11.5%), of which the latter two were present only in low levels in other treatments (*Alternaria*: O: 2.9%, U: 1.7%; *Aureobasidium*: O: 1.6%, U: 1.2%). Furthermore, the IM treatment contained higher percentages of unidentified taxa (17.4%) than the O (7.2%) or U (1.8%) treatments. Some of these genera were identified as having potentially important treatment effects by the random forest models (Table 2), and indeed there were higher *Zygophiala* percentages in U than the IM ($p = 0.002$) and O ($p = 0.03$) treatments, and higher percentages *Aureobasidium* in IM than in O or U treatments (both $p < 0.001$). However, the trend of higher *Penicillium* levels in IM than in O or U treatments was not significant (both $p > 0.11$). Thus, although skin tissues generally contained the same genera across treatments, the relative abundances of these genera were substantially altered by treatments.

Similarly, the relative abundance of taxa identified at the genus level also varied by treatment in pulp tissues. Pulp samples that received the O treatment were dominated by *Alternaria* (23.8%) and *Stomiopeltis* (21.7%), which were present only at low levels (<2.8%) in other treatments (Fig. 7). The U treatment was again dominated by *Zygophiala* (33.9%), which was largely absent in other treatments (<1.2%). Furthermore, the IM treatment again contained a larger percentage of unidentified taxa (27.8%) than other treatments (U: 13.3%, O: 6.9%), as well as a high relative abundance of *Penicillium* (25.1%), which was present in other treatment groups at lower relative abundances (U: 14.2%, O: 8.4%). The IM treatment also contained higher percentages of *Trichothecium* (5.8%) and *Exophiala* (3.06%) than other treatments (all <1%). Random forest models identified three of these taxa as variable across treatments: *Exophiala and Penicillium*, which were both higher in IM than O ($p = 0.04$ and $p = 0.005$,

respectively), and *Zygophiala*, which was higher in U than in IM and O treatments (both $p \leq 0.001$; Table 2).

Finally, seed samples contained the highest percentage of unidentified genera of all tissues (Fig. 7), particularly in the IM treatment (54.7%), but also in the O (24.7%) and U (22.2%) treatments. Compared to other tissue types, seeds appeared to be the least affected by treatment in terms of relative abundances of identifiable genera, as seeds were primarily dominated by *Penicillium* across treatments (IM: 14.2%, O: 35.8%, U: 43.9%). However, there were some differences in relative abundance; the U and O treatments contained higher percentages of *Zygophiala* (9.9% and 6.0%, respectively), compared to IM (<1%), and O also contained the highest relative abundance of *Paraconiothyrium* (8.6%) and *Exopassalora* (3.6%) than others (all <2.5% and <1%, respectively). Furthermore, the IM treatment contained a higher relative abundance of *Stomiopeltis* (4.2%) and *Cryptodiscus* (3.1%), while the U treatment contained higher percentages of *Filobasidium* (2.0%) and *Aspergillus* (1.7%) than other treatments (all <1%).

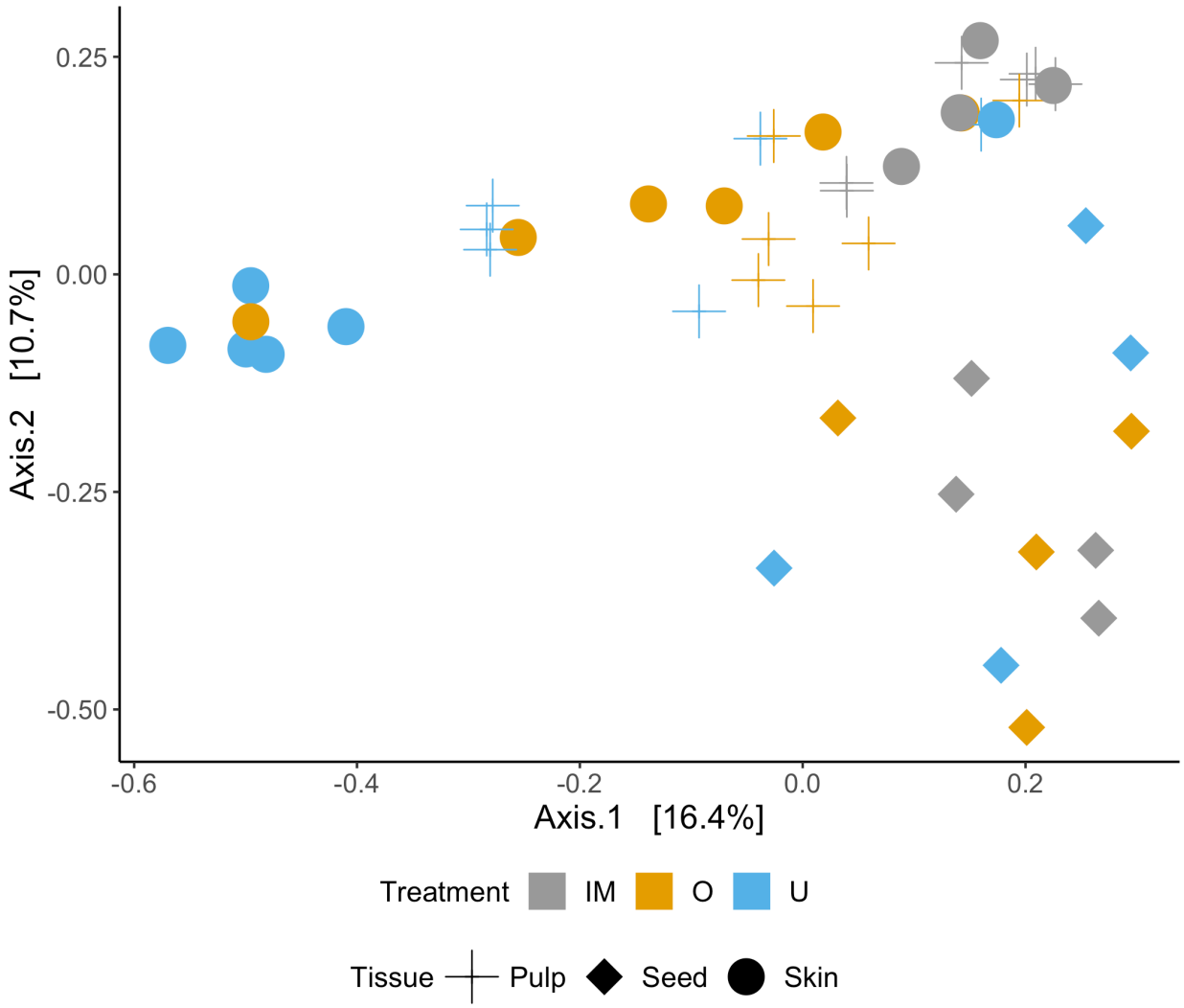

Figure 4. Principal coordinates analysis (PCoA) ordination plot of fruit fungal endophyte communities. These samples represent a subset of 'York' fruits that received the following treatments: IM (insecticide + antimicrobial), U (unsprayed), and O (holistic organic). Fruits were separated into skin, pulp, and seed samples. Distance was calculated using the Bray-Curtis dissimilarity matrix.

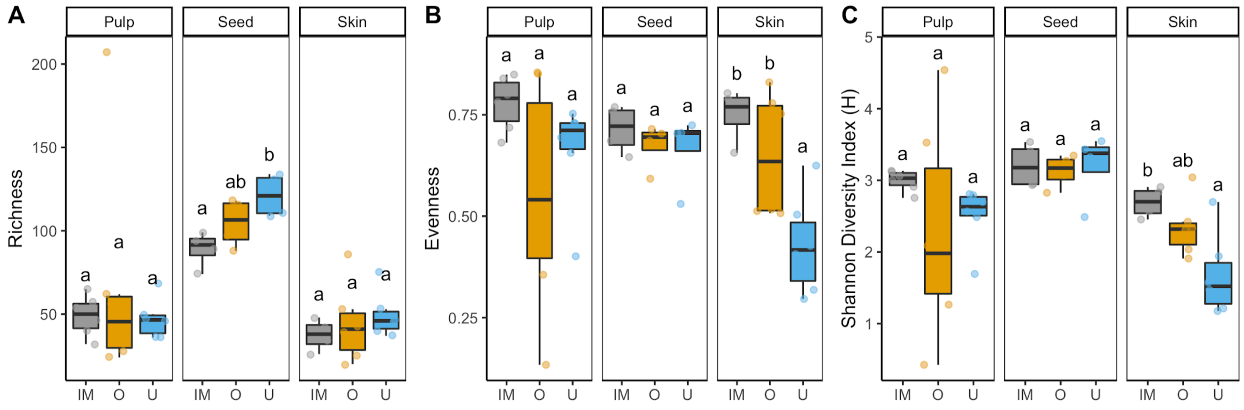

Figure 5. Fungal endophyte A) richness, B) evenness, and C) Shannon diversity index in apple fruit skin, pulp, and seed samples after IM (insecticide + antimicrobial), U (unsprayed), and O (holistic organic) treatments. All fruits belonged to the 'York' cultivar. Letters denote significant differences among treatment groups ($p < 0.05$).

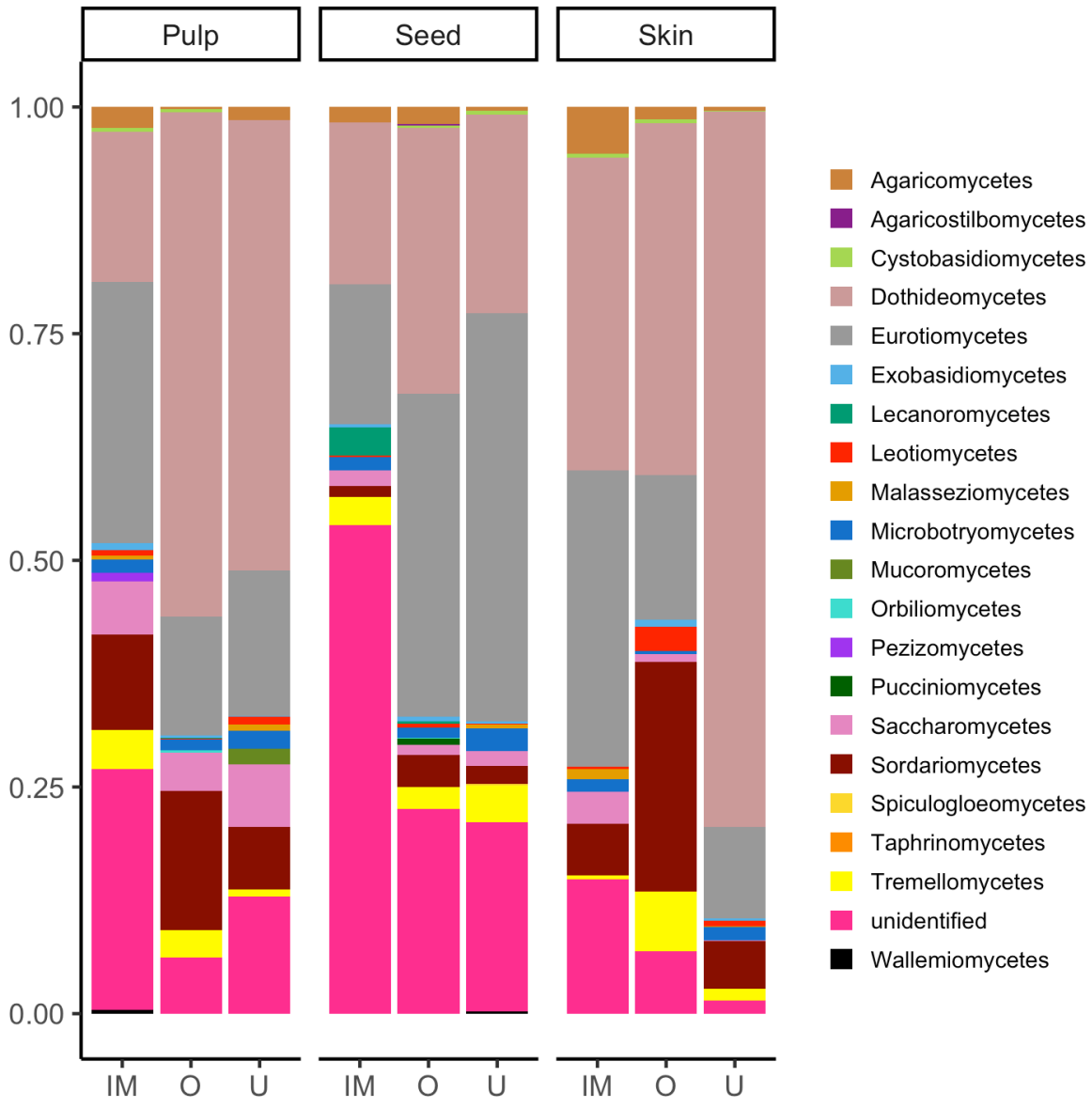

Figure 6. Relative abundance of fungal endophyte taxa at the class level in apple fruit skin, pulp, and seed samples after IM (insecticide + antimicrobial), U (unsprayed), and O (holistic organic) treatments. All fruits belonged to the 'York' cultivar.

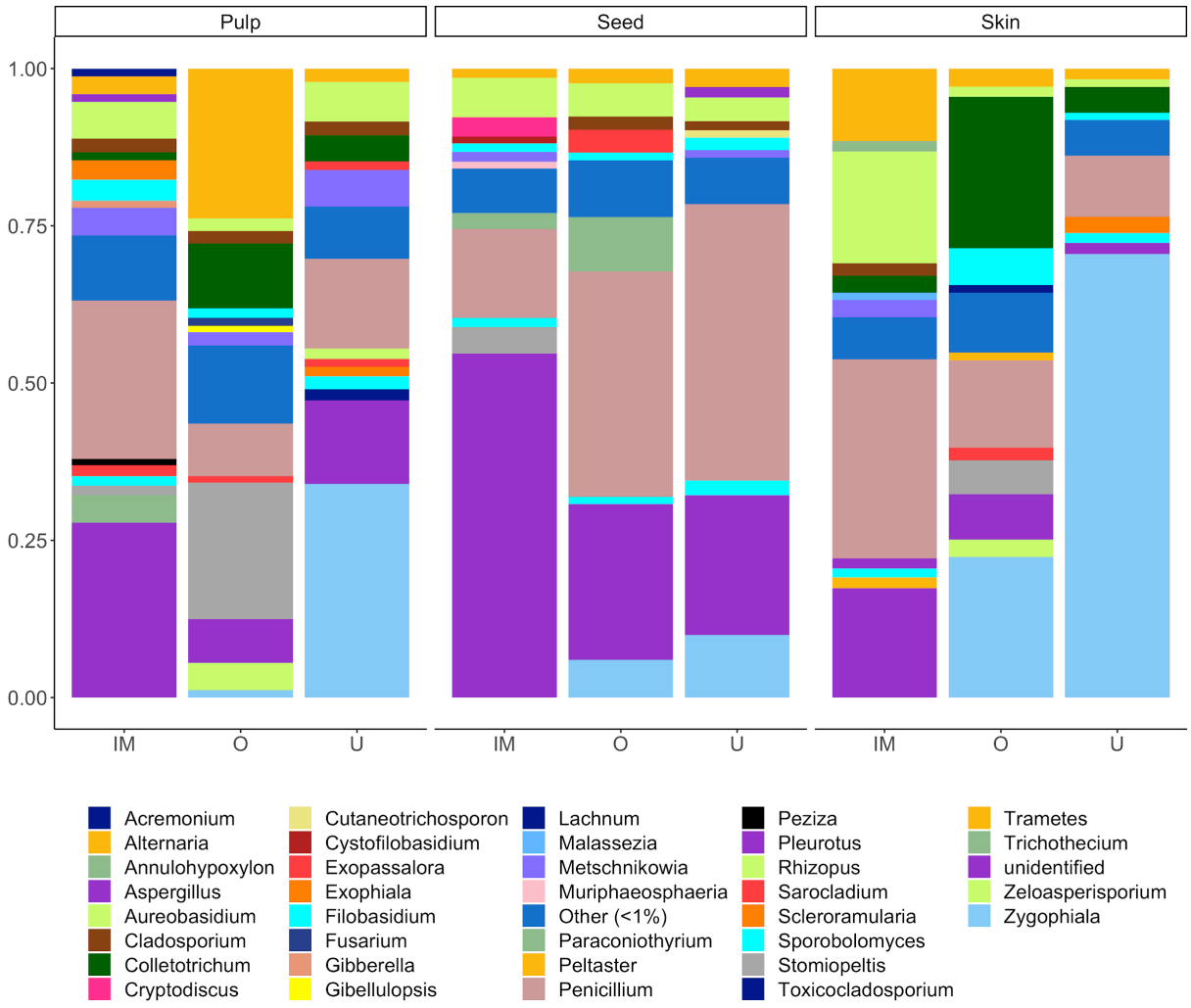

Figure 7. Relative abundance of fungal endophyte taxa at the genus level in apple fruit skin, pulp, and seed samples after IM (insecticide + antimicrobial), U (unsprayed), and O (holistic organic) treatments. All fruits belonged to the 'York' cultivar.

Table 2. Fungal endophytes identified as important by random forest models. The RF importance variable represents a standardized importance variable (Z-score) that has been assigned across all permuted random forest models. Separate models were used for each tissue type and taxonomic level.

Tissue Type	Endophyte	Taxonomic Level	RF variable importance
Skin	Dothideomycetes	Class	7.046017
Skin	Eurotiomycetes	Class	8.095249
Skin	Saccharomycetes	Class	10.314289
Pulp	Dothideomycetes	Class	6.9770431
Seed	Malasseziomycetes	Class	6.81005405
Skin	*Penicillium*	Genus	5.430660
Skin	*Zygophiala*	Genus	7.244484
Skin	*Aureobasidium*	Genus	3.44981813
Pulp	*Exophiala*	Genus	3.8790169
Pulp	*Penicillium*	Genus	4.4621738
Pulp	*Zygophiala*	Genus	6.4385818

Fruit phenolic chemistry

Overall, treatment effects on fruit phenolic chemistry varied by sampling season and tissue type. During the mid-season sampling period, treatments had no effect on the phenolic chemistry of unripe fruits, as there were no differences among treatment groups in phenolic compound composition (PERMANOVA; $p = 0.807$), total phenolic concentrations (all $p > 0.825$), or alpha diversity metrics such as richness, evenness, or Shannon diversity (all $p > 0.149$; Fig. 8).

In contrast to the mid-season sampling period, there were treatment effects on phenolic chemistry during late-season sampling, particularly for pulp samples. Treatments significantly influenced pulp phenolic compound composition (PERMANOVA; $p = 0.011$; Fig. 9), and pairwise comparisons revealed differences between the M and I treatments ($p = 0.03$; Fig. 9). Furthermore, the composition of phenolic compound classes tended to vary by treatment, as concentrations of benzoic acids were marginally higher in the I, U, and O treatments compared

to M (all $p < 0.07$), and concentrations of hydroxycinnamic acids were marginally higher in the O treatment than in the M treatment ($p = 0.09$; Fig. 10). At the individual compound level, chlorogenic acid (47.8-57.9%), procyanidin B2 (32.5-18.6%), and epicatechin (12.5%-8.2%) were present at the highest relative abundances in pulp tissues in all treatment groups (Fig. 11). In contrast to community composition, there were no differences in dispersion between treatments for pulp tissues (all $p > 0.46$; Fig. 9).

In addition to altering pulp phenolic composition, treatments also affected pulp alpha diversity metrics, as the O ($p = 0.0231$) and I ($p = 0.0183$) treatments contained higher levels of total phenolic concentrations than M (Fig. 12). Additionally, the I, O, and U treatments contained higher phenolic richness than M (all $p < 0.0266$), although there were no treatment effects on evenness ($p = 0.736$; Fig. 12), causing Shannon diversity values to only differ marginally between the I and M treatments ($p = 0.0585$).

Treatments also had some effect on skin phenolic chemistry during the late-season, altering overall phenolic compound composition (PERMANOVA; $p = 0.008$; Fig. 9), such that the IM and U treatments contained distinct compositions of phenolic compounds ($p = 0.05$). In terms of compound class composition, flavonols dominated skin samples, and tended to comprise a higher percentage of total concentrations in IM (60.8%) and M (59.8%) treatments compared to I (44.5%), U (40.6%), and O (39.4%), although this pattern was not significant (all $p > 0.126$; Fig. 10). Anthocyanins also tended to increase in IM (4.6%) and M (5.4%) treatments compared to the other three treatments (2.3-3.2%), such that the IM treatment contained marginally higher concentrations than U in the 'York' variety ($p = 0.09$). In contrast, hydroxycinnamic acids tended to compose a higher percentage of compound classes in the I, U, and O treatments (ranging from 11.1-14%) compared to IM and M (6.2 and 4.9%, respectively), as did dihydrochalcones (~4.5% in M and IM, ~7.5% in I, U, and O) and benzoic acids (~0.8% in M and IM, ~2% in I, U, and O; Fig. 10). At the individual compound level, hyperin (17.2-24.3% relative abundance), procyanidin B2 (13-15.5%), and avicularin (8.3-11.6%; Fig. 11) dominated across treatments. In contrast to phenolic compound composition, there were no significant differences in dispersion of treatment groups in skin samples (all $p > 0.23$; Fig. 9).

Although treatments altered skin phenolic composition, they only marginally affected skin total phenolic concentrations and diversity metrics, in contrast to pulp samples. Specifically, total phenolic concentrations were marginally affected by treatments in skin samples ($p =$

0.0886), such that IM-treated fruits tended to contain higher phenolic concentrations than other treatments (Fig.12), and skin Shannon diversity was marginally higher in the I treatment relative to M ($p = 0.079$).

Some individual phenolic compounds were upregulated in the treatment groups that did not receive antimicrobials and were thus exposed to higher fungal damage. An unidentified compound designated as "compound D" and classified as a benzoic acid based on its UV spectra was selected as an important compound in random forest models (Table 3), and was strongly upregulated in the non-antimicrobial treatments across sampling season and tissue type. Specifically, this compound appeared in higher concentrations in the I, U, and O treatments compared to M and IM for mid-season skin (all $p > 0.0175$; Fig. 13A), mid-season pulp (all $p < 0.0261$), except for I, which was higher than M but not IM ($p = 0.363$; Fig. 13B), late-season skin (all $p < 0.002$; Fig. 13C), and, at least marginally, late-season pulp samples (all $p < 0.0623$; Fig. 13D). Additionally, chlorogenic acid, a hydroxycinnamic acid, was selected as an important variable for late-season skin and pulp samples (Table 3), and was present in higher concentrations in I, U, and O treatments than the M treatment for both skin and pulp samples (all $p < 0.21$, Fig. 14).

Although other compounds were identified as important by the random forest models (Table 3), they displayed weak, inconsistent patterns across sampling season, cultivar, and tissue types (Fig 15). In late-season 'Golden Delicious' skin samples, there were higher concentrations of compound C, a benzoic acid, in the I treatment compared to the IM ($p = 0.0491$), M ($p < 0.001$) and O ($p < 0.001$) treatments, and lower or marginally lower concentrations in the M treatment than U ($p = 0.0169$) and IM ($p = 0.0715$) treatments (Fig. 15A). Furthermore, in late-season pulp samples, compound C was found in higher concentrations in the I treatment relative to M ($p = 0.00658$), IM ($p = 0.0437$), and O treatments ($p = 0.0695$; Fig. 15B). Although 'York' samples showed a trend of elevated concentrations of compound C in the I, U, and O treatments compared to the M and IM treatments (Fig.15A-B), this was not significant (all $p > 0.155$). Additionally, only in 'Golden Delicious' fruits, a benzoic acid designated as compound P was present in higher concentrations in the I treatment compared to O in mid-season skin samples ($p = 0.0225$; Fig. 15C), a benzoic acid designated as compound H displayed higher concentrations in I than M, IM, and O treatments in late-season skin samples (all $p < 0.0374$; Fig. 15D), and a flavan-3-ol identified as procyanidin B2 was present in higher concentrations in IM ($p = 0.0193$)

and marginally higher in M ($p = 0.0926$) than O in late-season pulp samples (Fig.15F). Additionally, concentrations of quercitrin, a flavonol, were elevated in the IM treatment relative to O ($p = 0.00944$; Fig. 15E).

When comparing overall patterns of phenolic chemistry across sampling seasons, phenolic concentrations and diversity metrics tended to be higher in unripe fruits collected during the mid-season sampling period compared to the late-season. Specifically, mid-season fruits contained higher levels of skin total phenolic concentrations ($p < 0.001$), richness ($p < 0.001$) and Shannon diversity ($p = 0.0183$), although lower evenness ($p < 0.001$), compared to the late-season ($p < 0.001$; Fig. 16). Furthermore, mid-season pulp samples contained higher evenness ($p < 0.001$) and Shannon diversity ($p < 0.001$), but showed no difference in richness between sampling seasons ($p = 0.95$). In contrast to skin samples, however, the total phenolic concentrations in pulp samples increased in late-season ripe fruits compared to mid-season unripe fruits ($p < 0.001$; Fig. 16A).

Finally, cultivars sometimes displayed different patterns of phenolic chemistry. ‘York fruits tended to contain higher overall phenolic concentrations and alpha diversity metrics, as they contained significantly higher total phenolic concentrations and richness in mid-season seed samples than ‘Golden Delicious’ (both $p < 0.001$), but lower evenness ($p = 0.0475$; Fig. 8). Furthermore, ‘York’ fruits contained higher late-season skin ($p < 0.001$) and pulp ($p = 0.0239$) phenolic richness than ‘Golden Delicious’, but lower evenness (both $p < 0.001$). However, ‘Golden Delicious’ contained several compounds that displayed treatment effects that did not occur in ‘York’ fruits (Table 3; Fig. 15), and compound D, although upregulated in both cultivars by treatment, was present in higher concentrations in the ‘Golden Delicious’ cultivar than in ‘York’ (all $p < 0.0346$; Fig. 13).

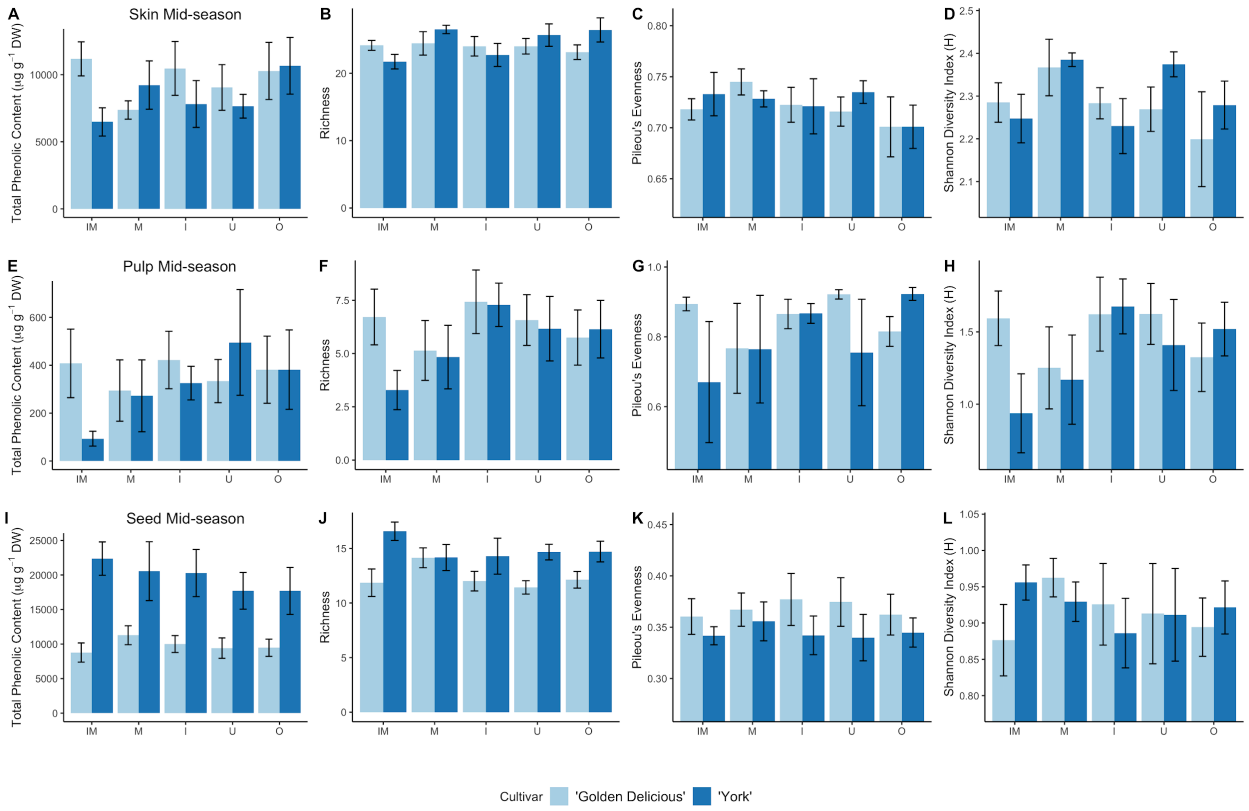

Figure 8. Mid-season total phenolic content (µg g^{-1} DW), richness, evenness, and Shannon diversity in skin (A-D), pulp (E-H), and seed (I-L) tissues after application of IM (insecticide + antimicrobial), M (antimicrobial), I (insecticide), U (unsprayed), and O (holistic organic) treatments. Bars indicate standard error.

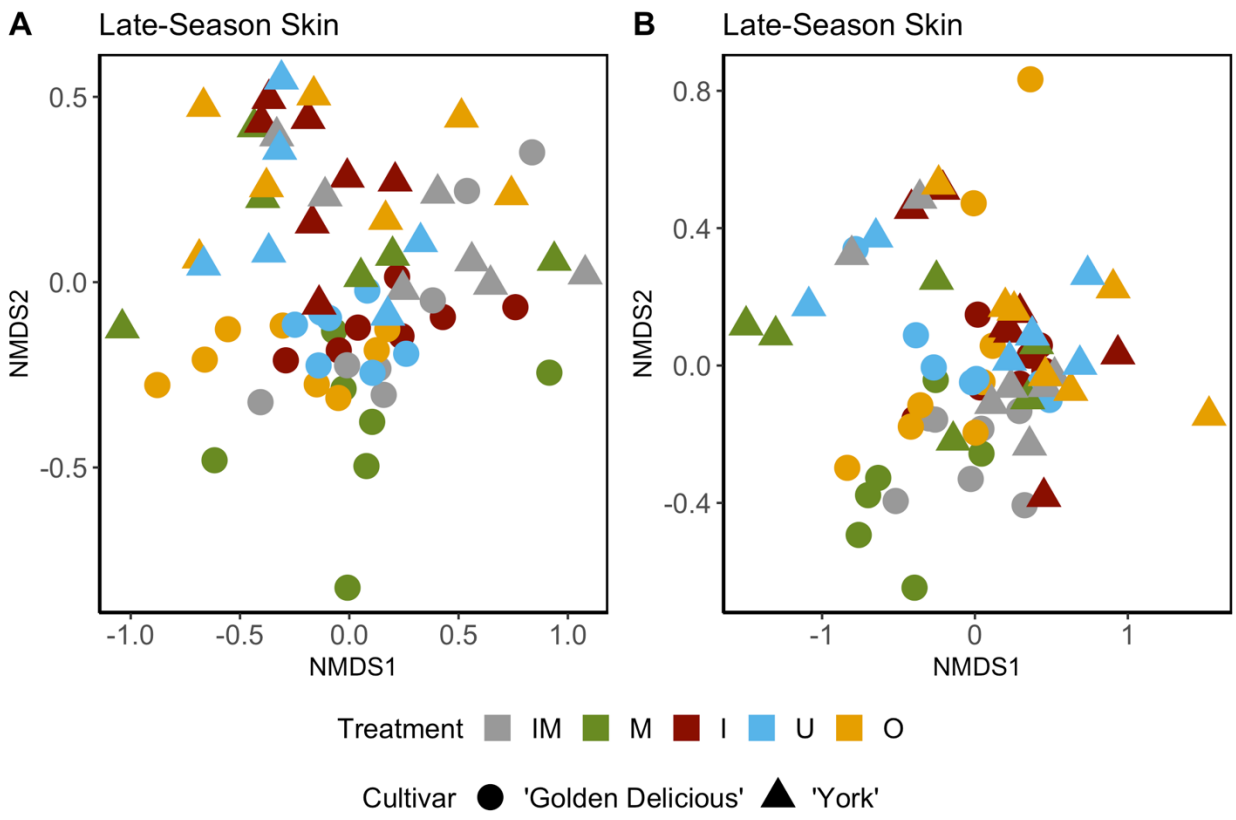

Figure 9. NMDS ordination plot of phenolic compounds in late-season A) skin and B) pulp fruit tissues after application of the following treatments: IM (insecticide + antimicrobial), M (antimicrobial), I (insecticide), U (unsprayed), and O (holistic organic).

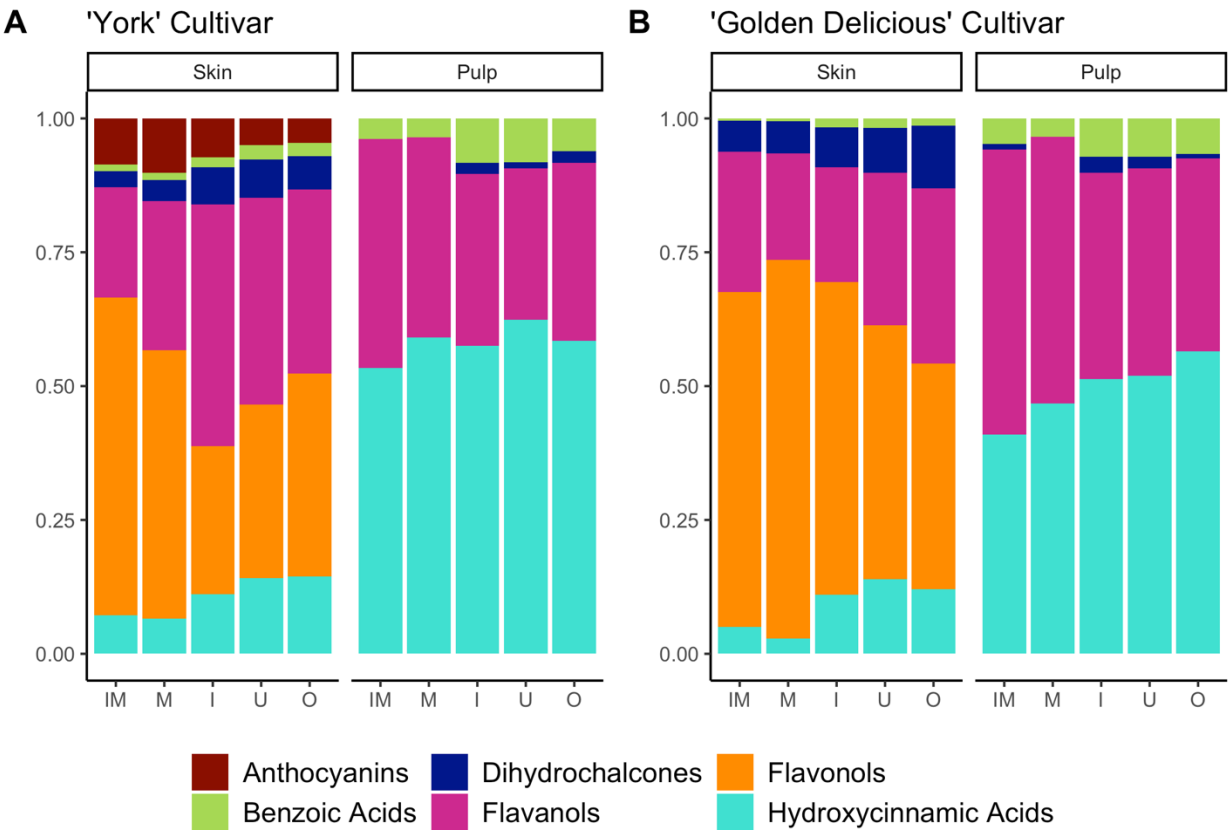

Figure 10. Relative abundance of phenolic compound classes in late-season apple fruit skin and pulp samples after IM (insecticide + antimicrobial), M (antimicrobial), I (insecticide), U (unsprayed), and O (holistic organic) treatments by ‘York’ and ‘Golden Delicious’ cultivars.

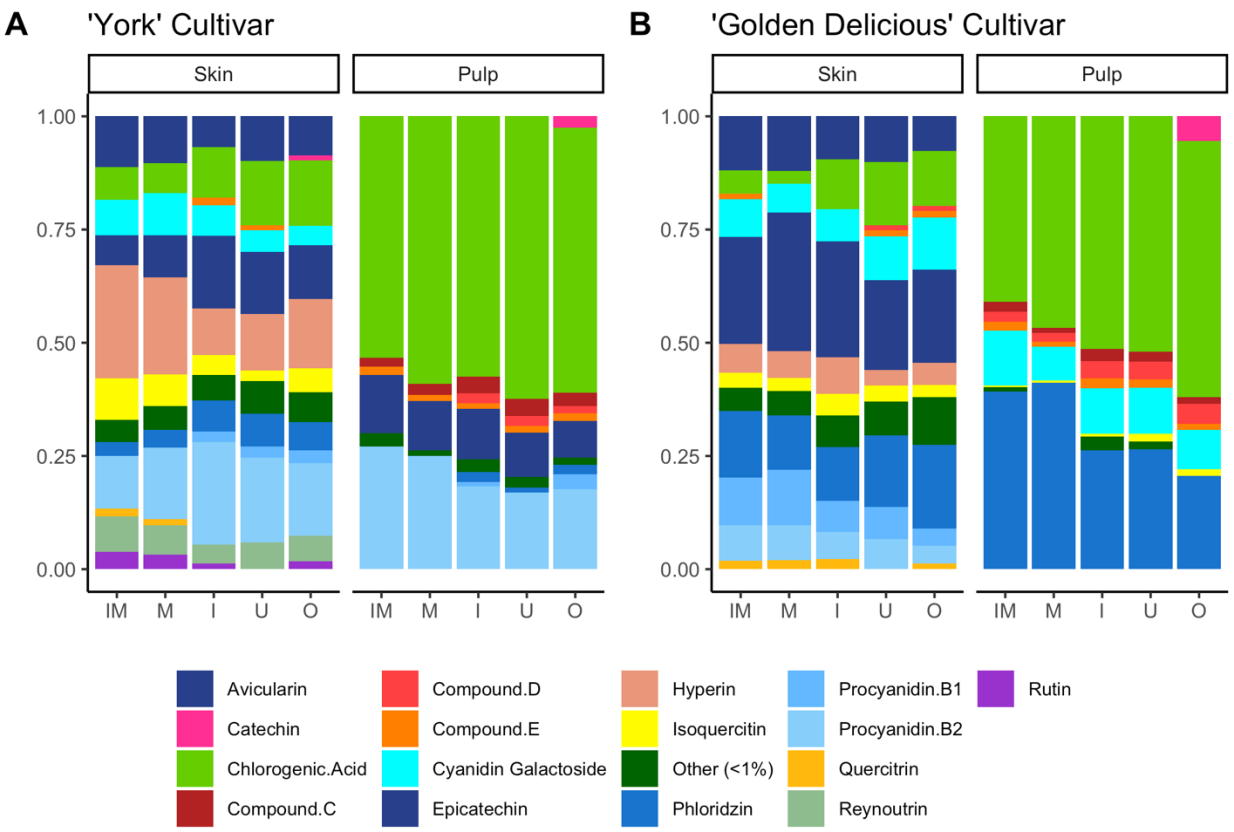

Figure 11. Relative abundance of phenolic compounds in late-season apple fruit skin and pulp samples after IM (insecticide + antimicrobial), M (antimicrobial), I (insecticide), U (unsprayed), and O (holistic organic) treatments by ‘York’ and ‘Golden Delicious’ cultivars.

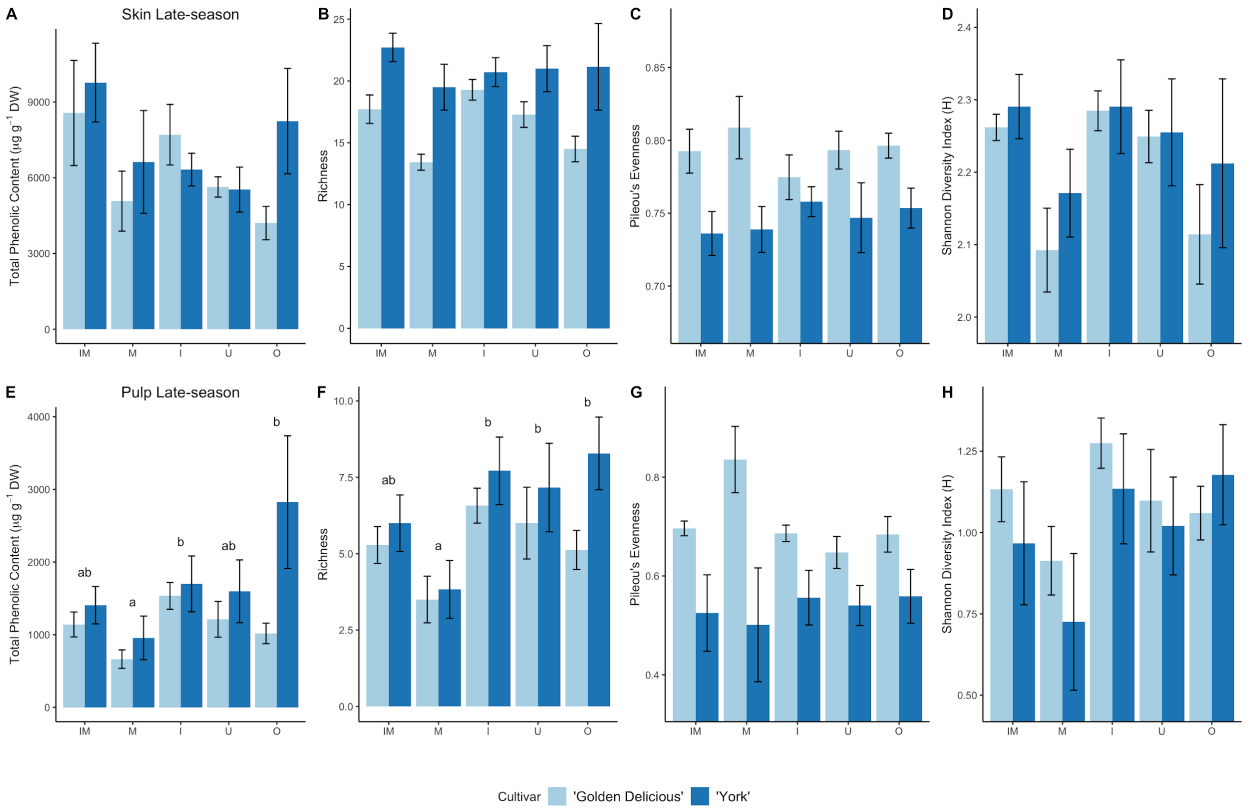

Figure 12. Late-season total phenolic content (µg g^{-1} DW), richness, evenness, and Shannon diversity in skin (A-D) and pulp (E-H) tissues after application of IM (insecticide + antimicrobial), M (antimicrobial), I (insecticide), U (unsprayed), and O (holistic organic) treatments. Bars indicate standard error. Letters denote significance among treatment groups ($p < 0.05$) across both cultivars.

Table 3. Phenolic compounds identified as important by random forest models. The RF importance variable represents a standardized importance variable (Z-score) that has been assigned across all permuted random forest models. Separate models were used for each tissue type and sampling season. Cultivars were examined using both a combined model (Both) and separate 'Golden Delicious' (GD) or 'York' (Y) models.

Compound	Compound Class	Sampling Period	Tissue	Cultivar	RF variable importance
Compound D	Benzoic acid	Mid-season	Skin	Both	10.39215901
		Mid-season	Pulp	Both	16.7712762
		Late-season	Skin	Both	11.0332983
		Late-season	Pulp	Both	18.67153556
Chlorogenic acid	Hydroxycinnamic acid	Late-season	Skin	Both	5.7990445
		Late-season	Pulp	Both	3.14316063
Compound C	Benzoic acid	Late-season	Skin	GD	5.28311857
		Late-season	Pulp	GD	8.85832797
Compound P	Benzoic acid	Mid-season	Skin	GD	3.85028737
Compound H	Benzoic acid	Late-season	Skin	GD	5.12984790
Quercitrin	Flavonol	Late-season	Skin	GD	8.24021526
Procyanidin B2	Flavanol	Late-season	Pulp	GD	11.43490230

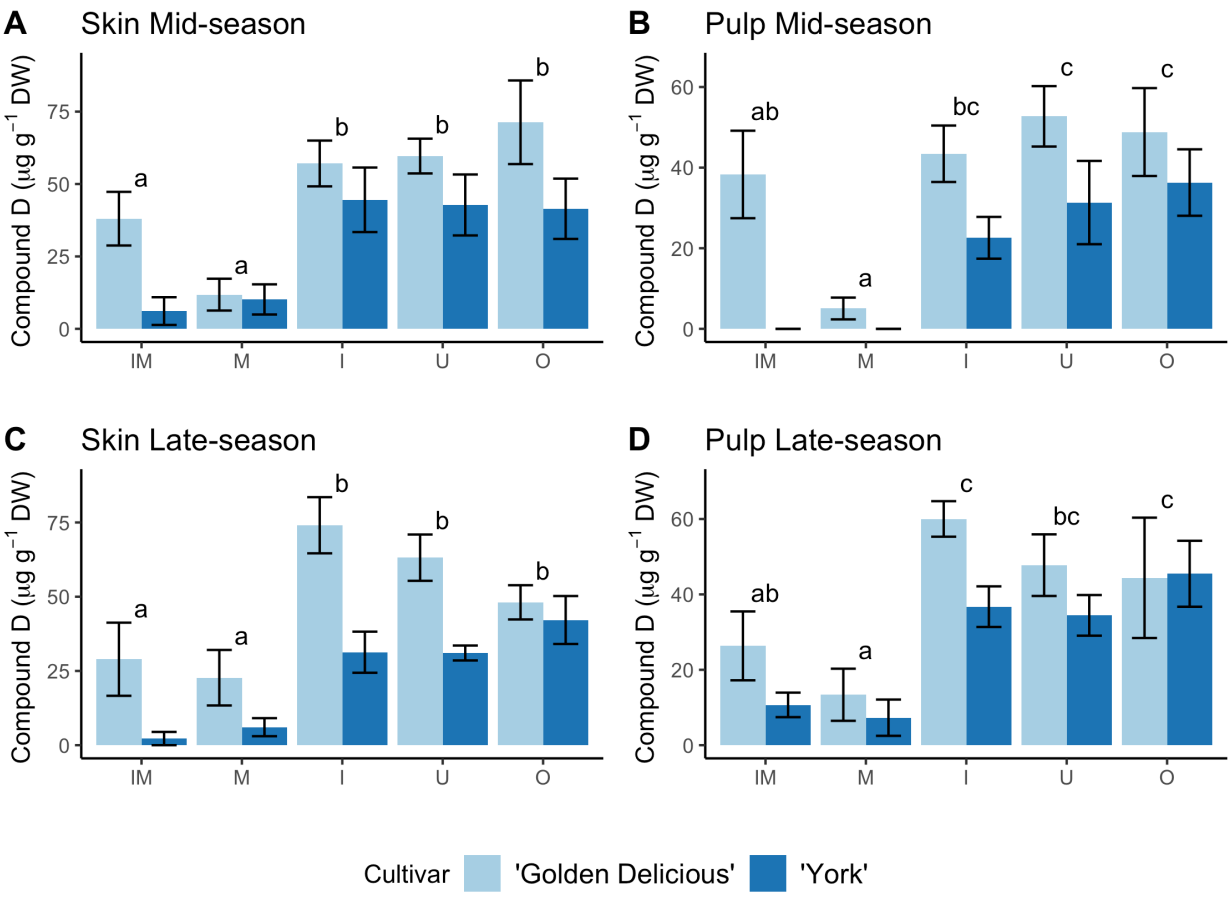

Figure 13. Total concentrations (µg g^{-1} DW) of compound D in A) mid-season skin, B) mid-season pulp, C) late-season skin, and D) late-season pulp tissues after application of IM (insecticide + antimicrobial), M (antimicrobial), I (insecticide), U (unsprayed), and O (holistic organic) treatments. Bars indicate standard error. Letters denote significance among treatment groups ($p < 0.05$) across both cultivars.

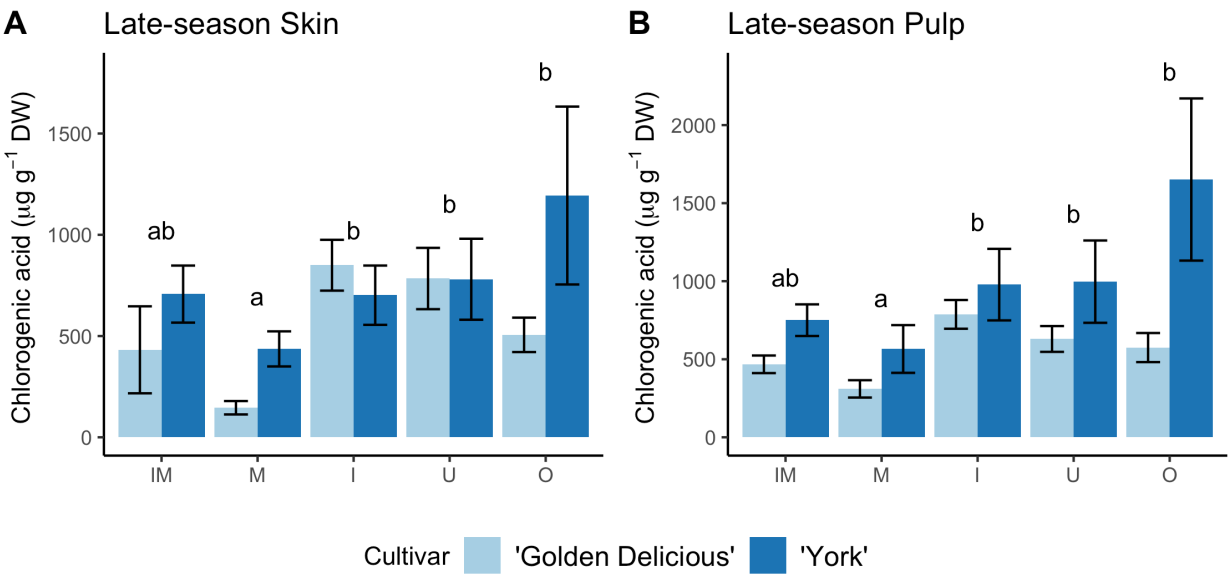

Figure 14. Total concentrations (µg g^{-1} DW) of chlorogenic acid in A) late-season skin and B) late-season pulp tissues after application of IM (insecticide + antimicrobial), M (antimicrobial), I (insecticide), U (unsprayed), and O (holistic organic) treatments. Bars indicate standard error. Letters denote significance among treatment groups ($p < 0.05$) across both cultivars.

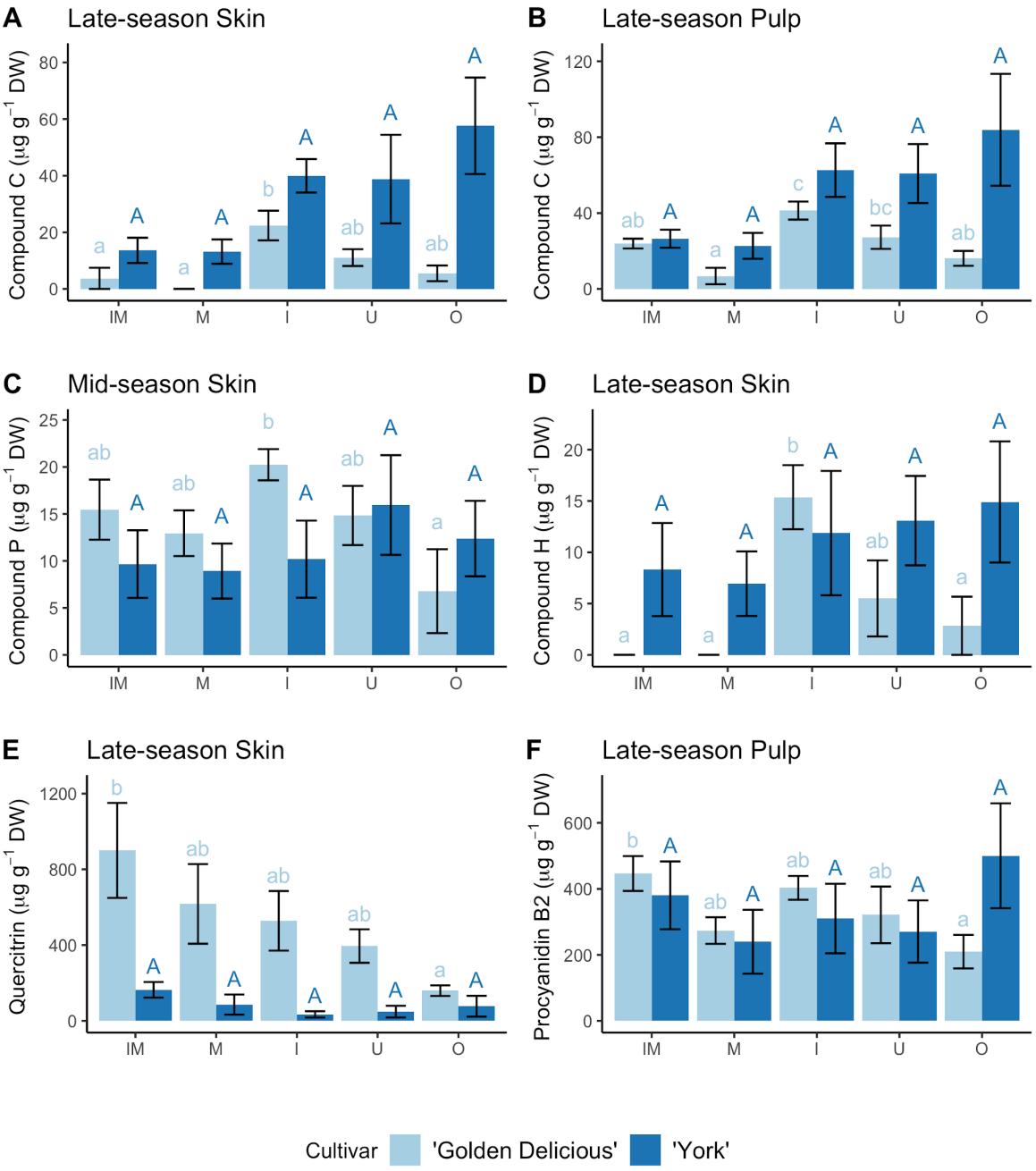

Figure 15. Total concentrations (µg g^{-1} DW) of A) compound C in late-season skin, B) compound C in late-season pulp, C) compound P in mid-season skin, D) compound H in late-season skin, E) quercitrin in late-season skin, and F) procyanidin B2 in late-season pulp tissue samples after application of IM (insecticide + antimicrobial), M (antimicrobial), I (insecticide), U (unsprayed), and O (holistic organic) treatments. Bars indicate standard error. Letters denote significance among treatment groups ($p < 0.05$) assessed separately for cultivars. Letter colors correspond to cultivar color.

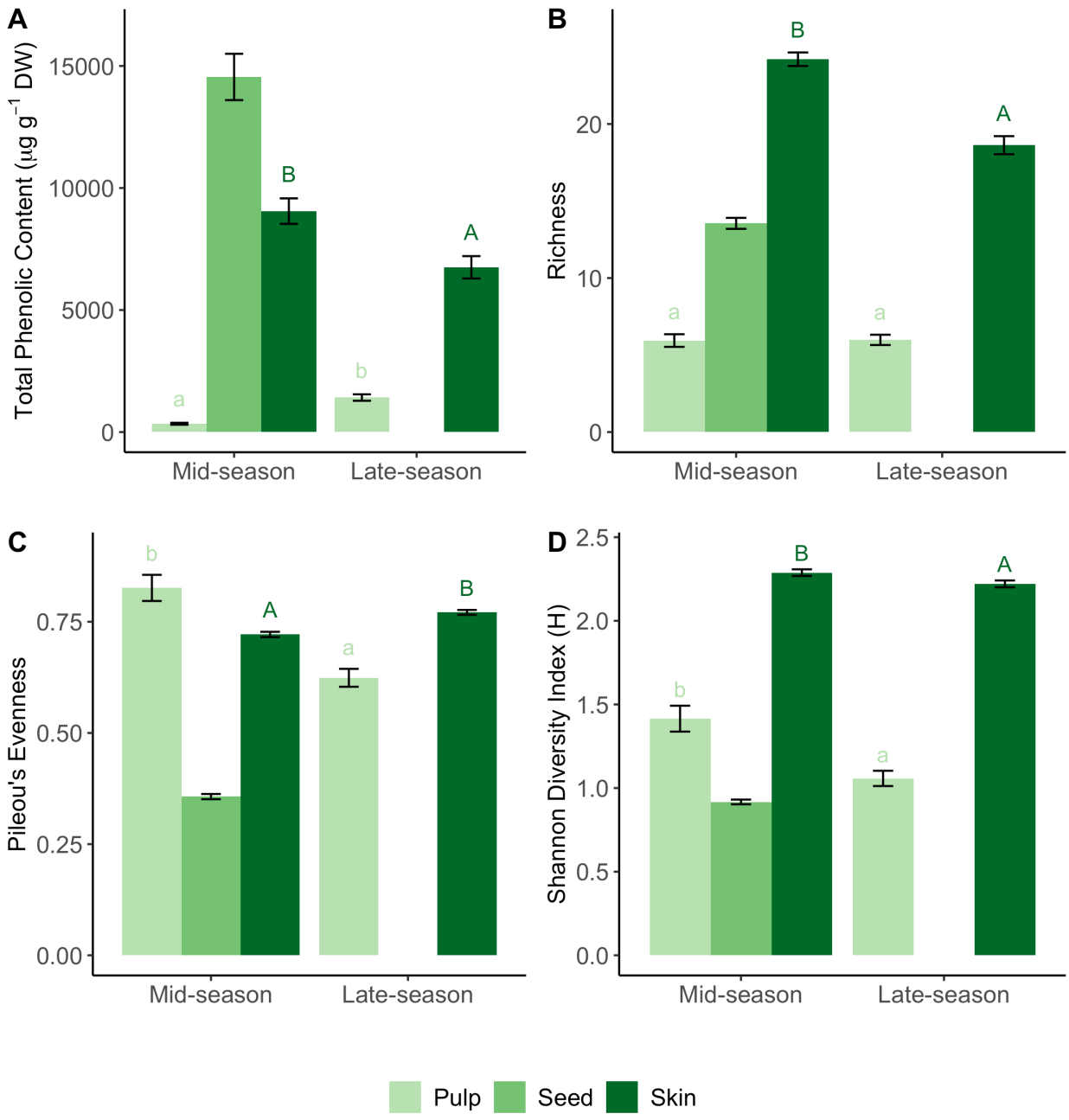

Figure 16. Levels of A) total phenolic content (µg g^{-1} DW), B) richness, C) evenness, and D) Shannon diversity in skin, pulp, and seed samples combined across treatments for mid and late-season sampling periods. Bars indicate standard error. Letters denote significance between sampling seasons ($p < 0.05$) assessed separately for tissue types. Letter colors correspond to tissue colors.

Phenolic chemical diversity vs. fungal endophyte diversity

There was a positive relationship between phenolic chemical diversity and fungal endophyte diversity within individual fruits, but only for one diversity metric (Shannon diversity) in pulp tissues (Fig. 17A). However, there were non-significant positive trends in most other diversity metrics in both skin and pulp tissues (Fig. 17). Specifically, pulp phenolic Shannon diversity increased with endophyte diversity ($p = 0.03$, $R^2 = 0.21$, slope = 0.26), and there were positive trends in pulp richness ($p = 0.15$, $R^2 = 0.12$, slope = 0.028) and evenness ($p = 0.15$, $R^2 = -0.025$, slope = 0.34; Fig. 17). Although there was no relationship in skin samples between endophyte and phenolic diversity ($p = 0.90$, Adjusted $R^2 = -0.20$, slope = 0.023) or evenness ($p = 0.89$, $R^2 = -0.14$, slope = -0.016), there was a slight trend of a positive increase of phenolic richness with endophyte richness, albeit non-significant ($p = 0.38$, $R^2 = -0.10$, slope = 0.10; Fig. 17). When each treatment group was evaluated separately, there were no significant effects (all $p > 0.11$).

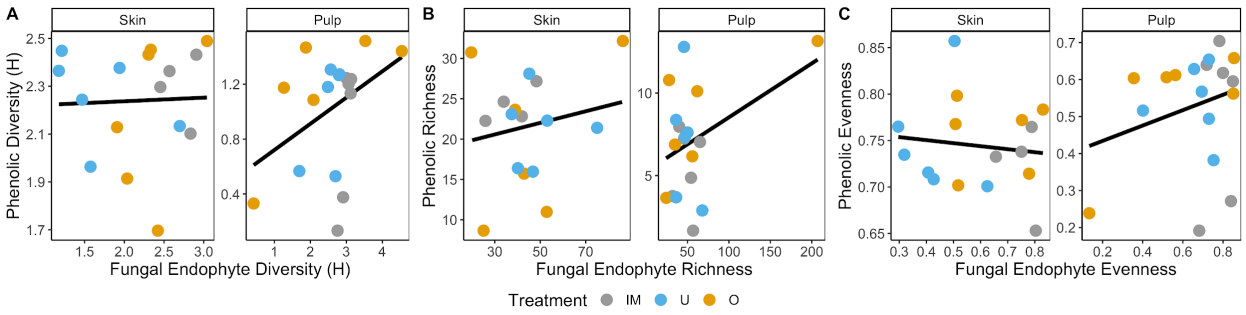

Figure 17. Fungal endophyte and phenolic A) Shannon diversity, B) richness, and C) evenness after IM (insecticide + antimicrobials), U (unsprayed), and O (holistic organic) treatments. Points were fit with a linear regression and were analyzed separately for skin and pulp tissues.

Discussion

We used five treatments, IM (insecticide +antimicrobials), M (antimicrobials), I (insecticide), U (unsprayed), and O (holistic organic), to create distinct communities of biotic interactions, and characterized these communities through fruit damage evaluations and sequencing of fruit fungal endophyte communities. We found that the antimicrobial treatments strongly affected fruit communities, as both visible fungal damage and fungal endophyte

community composition varied between antimicrobial and non-antimicrobial treated fruits (Fig. 1,2,4). Specifically, the U and O treatments were dominated by sooty blotch and flyspeck pathogens (SBFS) in contrast to the IM treatment, particularly in skin tissues, which may explain the higher endophyte diversity found in IM skin compared to U ($p = 0.02$; Fig. 5). However, the O treatment was also distinct in both endophyte composition (Fig. 4) and fruit russeting levels (Fig. 3). We then assessed the effects of these communities on fruit phenolic compound diversity and composition, which varied by sampling season, cultivar, and fruit tissue type. However, non-antimicrobial treated fruits (I, U, and O) that were exposed to higher fungal damage levels contained higher or marginally higher richness and total concentrations in late-season pulp tissues (Fig. 12), increased concentrations of chlorogenic acid in late-season pulp and skin tissues (Fig. 14), as well as increased concentrations of compound D, an unknown benzoic acid, across tissue type and sampling season (Fig. 13), indicating an induced defensive response to fungal damage. Finally, when comparing the relationship between endophyte and phenolic diversity within individual fruits, we found that phenolic Shannon diversity increased with fungal endophyte Shannon diversity in pulp tissues, but not skin tissues (Fig. 17). Overall, these results suggest that the relationship between biotic diversity and plant chemical diversity is context-dependent, and may vary by season, tissue type, or cultivar.

Fruit Damage Evaluations

Overall, fruit damage evaluations revealed that treatments did create distinct levels of biotic interactions, and were particularly effective at establishing variable amounts of fungal pathogen damage, but were less effective at creating differences in insect damage. During both sampling seasons, antimicrobials limited fungal damage, both overall and for sooty blotch and flyspeck specifically (all $p < 0.0278$; Fig. 1E-F; Fig. 2). In contrast, insect damage was only lower in insecticide-treated fruits during the mid-season sampling period (all $p < 0.0979$), but this trend disappeared in the late-season (Fig. 1C-D). This could be an effect of timing, as fruit oviposition from pests such as apple maggot (*Rhagoletis pomonella*) and plum curculio (*Conotrachelus nenuphar*) typically occurs during the mid-season, and fruits that are infected with larvae often drop to the ground, allowing pupae to emerge in soil (Glass 1966; Schmidt et al. 2016). Indeed, we observed that many of the trees that received no pesticide treatments (i.e., the U and O treatments) dropped a substantial amount of fruit prior to late-season sampling,

perhaps limiting our ability to detect insect damage. However, fruits also experienced overall lower levels of insect damage than fungal (Fig. 1); in addition to sooty blotch and flyspeck, we observed damage from such fungal pathogens as cedar-apple rust (*Gymnosporangium juniperi-virginianae*), bitter rot (*Colletotrichum sp.*), and white rot (*Botryosphaeria dothidea*). In contrast, insect damage levels were low, and were driven primarily by plum curculio, while destructive Lepidoptera such as the codling moth (*Cydia pomonella*), for instance, were largely absent. This high fungal load could have been driven by environmental conditions, as it was an unusually wet summer, or it may also be that after years of conventional pesticide treatments in the orchard, the fungal pathogens were able to re-establish more quickly than the insect pests. Regardless, treatments created community differences more effectively for fungal pathogens than insect pests.

The fungal pathogens responsible for the majority of fruit damage were sooty blotch and flyspeck. These infections are caused by a complex of fungi that grows on the fruit exterior and that may completely cover the fruit surface, which was observed in fruits that received the I, O, and U treatments (Fig. 2). Although these fungi may cause severe cosmetic damage to the fruit, forcing growers to convert infected fruit for use in fruit juice processing instead of fresh produce and lose an estimated 90% of fruit value (Gleason et al. 2011), these pathogens cause no tissue damage to the fruit. In fact, they have been characterized as ectophytes that invade nonliving plant surfaces, embedding in the wax and cuticle layers and gaining nutrients from sugar-containing exudates leached from the fruit, but do not invade living plant cells (Williamson and Sutton 2000; Gleason et al. 2019). Thus, these pathogens may not induce a plant defensive response at all. Indeed, when compared to parasitic fungal species that invade living tissues, several species of the sooty blotch-flyspeck complex were shown to contain smaller genomes with substantially reduced percentages of genes associated with host invasion (Xu et al. 2017). Thus, these species may evade host plant detection entirely due to their minimal production of elicitors that would trigger a plant immune response, and have even been referred to as “stealth pathogens” that are able to reside undetected by host species (Gleason et al. 2019). However, the effect of sooty blotch and flyspeck infection on plant induced defenses has not been directly investigated. Furthermore, even if the presence of these pathogens does not directly impact plant chemistry, there may still be indirect effects on fruit traits through alterations to the fruit surface environment and potential cascading effects on biotic community composition. For example,

although these species have a significantly reduced genome, they have been shown to retain or increase their ability to degrade epicuticular waxes, to produce melanin, which protects against stress from UV irradiation and high temperatures, and to produce lysozymes, which defend against competing bacteria (Gleason et al. 2019; Xu et al. 2017). Thus, sooty blotch and flyspeck may potentially have minimal effects on inducing plant defenses, but they may also have a large impact in shaping carposphere community structure by altering the environmental conditions of the fruit surface or by directly competing against other pathogens.

Interestingly, treatments influenced the amount of russeting observed on the fruit, which was found to be higher in the holistic organic organic treatment (O) compared to all others in both the mid and late-season sampling periods (all $p < 0.001$; Fig. 3). Russeting is a fruit skin malformation that occurs when the cuticle cracks and a corky layer of periderm forms underneath (Khanal et al. 2012). This rough surface is impermeable to water and nutrients, preventing sooty blotch and flyspeck growth on this region (Williamson and Sutton 2000), which may explain their decreased infection levels in the O treatment compared to U and I (all $p < 0.01$; Fig. 3). It is unclear exactly what causes russeting in apples, but it has been linked to factors such as high moisture levels (Knoche and Grimm 2008), UV light exposure (Noè and Eccher 1996), and invasion by a variety of organisms, including the fungal pathogen *Aureobasidium pullulans*, the yeast *Rhodotorula glutinis* (Heidenreich et al. 1997), the apple rust mite *Aculus schlechtendal* (Easterbrook and Fuller 1986), and the fungal pathogen powdery mildew (*Podosphaera leucotricha*; Daines et al. 1984). It has been suggested that these organisms actively degrade the fruit cuticle, a capability that has been observed in *A. pullulans* (Goffinet et al. 2002), and thus expose the epidermal cells to environmental stressors, resulting in russeting (Gildemacher et al. 2006). Regardless of the mechanism, here, the higher russeting levels in the O treatment is likely due to increased colonization of microorganisms on fruit surfaces, driven either by the effective microbes added during treatment application or by the attraction of microbial taxa to the organic amendments included in the treatment. This illustrates the complexity of using microbial inoculants as biocontrol; the amendments designed to promote fruit defenses in Phillips' treatment appeared to have unexpected impacts on fruit structure. However, although cosmetically unappealing, russeting is not necessarily detrimental for consumers; one study found that triterpene-caffeates produced only in russeted regions of apple skins elicited health-promoting anti-inflammatory effects (Andre et al. 2013).

Fruit damage levels did vary by cultivar, as 'York' fruits sustained higher damage levels than 'Golden Delicious' (Fig. 1), including damage from flyspeck and sooty blotch (both $p < 0.003$; Fig. 2). Flyspeck and sooty blotch infection success has been shown to vary by cultivar, possibly due to differences in cuticle structures, as these pathogens prefer permeable cuticles that may leach more sugar (Williamson and Sutton 2000). Furthermore, russeting occurred primarily in 'Golden Delicious' fruits ($p < 0.002$) and was absent in 'York' (Fig. 3); this cultivar-level variability has been well-documented and has been suggested to have a genetic basis (Falginella et al. 2015). In short, some cultivars are more resistant to invasion than others, potentially due to underlying structural differences as well as differing chemical and physical responses to antagonists.

Fungal Endophyte Community

At the microbiome level, treatments did create distinct communities of fungal endophytes, as IM, U and, O-treated fruits differed in community composition ($p = 0.001$; Fig. 4). However, treatment impacts on diversity and richness were inconsistent across tissue type; while IM-treated fruits contained higher skin diversity than U ($p = 0.02$), the pattern was reversed in seed richness ($p = 0.001$), and O-treated fruits displayed an intermediate trend both times (Fig. 5). The increased skin diversity in the IM treatment may be due to different infection levels of the dominant fungal pathogens sooty blotch and flyspeck (SBFS), which completely covered fruit surfaces in the U and O treatments, but were only present in small patches in the IM treatment (Fig. 2). This SBFS dominance in the U treatment was confirmed by sequencing of fungal endophyte taxa, as unsprayed fruit skin tissues contained the highest percentages of the class Dothideomycetes (all $p < 0.01$; Fig. 6), which contains *Zygophiala*, a genus in the SBFS complex that comprised over 70% of relative abundance in U skin tissues (Fig. 7). Thus, SBFS pathogens likely outcompeted other fungal endophytes in unsprayed skin tissues, causing a decrease in endophyte diversity. In contrast to U, the O and IM treatments were not as dominated by *Zygophiala*. The IM treatment contained the highest relative abundance of the genus *Penicillium*, which includes *Penicillium expansum*, an important pathogen in post-harvest apple disease (Wang et al. 2018). Also prevalent in the IM treatment were the genera *Alternaria*, which includes *Alternaria alternata*, another apple pathogen (Grove et al. 2003; Harteveld et al. 2013), and *Aureobasidium pullulans*, which has been identified as a potential biocontrol agent in

postharvest apple diseases (Kheireddine et al. 2018), as well as the class Saccharomyces, which contains yeasts. The dominance of *Penicillium* and *Alternaria* in the IM treatment is consistent with the findings of Abdelfattah et al. (2016), who found these two genera to be highly prevalent in the skin tissues of store-bought apples. Furthermore, the increased relative abundance of certain taxa and overall higher diversity in the IM treatment suggests that some taxa may be resilient to pesticides; indeed, both *Penicillium* and *Alternaria* have been documented to develop resistance to fungicides (Avenot et al. 2008; Kinay et al. 2007). Thus, while some microbiota may be sensitive to pesticide application, others may increase in abundance due to increased resource availability from the disappearance of pesticide-sensitive microbes or potentially from the pesticides themselves. Indeed, several species of yeasts have been shown to increase in abundance in response to fungicide or insecticide application (Čadež et al. 2010; Moulas et al. 2013). Finally, similar to the U treatment, the O treatment also contained high levels of *Zygophiala* and *Penicillium*, in addition to *Colletotrichum*, a genus which contains such apple pathogens as bitter rot (*Colletotrichum gloeosporioides*). Thus, the dominant taxa across treatments were generally pathogens and prospective biocontrol agents; it should be noted, however, that this trend may merely be a result of their agricultural importance, leading to a disproportionate amount of taxonomic information on these species, while a substantial amount of endophyte diversity remains unidentified. These unidentified taxa were most highly abundant in the IM treatment, which also contained the highest skin diversity (Fig. 7; Fig. 5C).

Pulp tissues contained a similar endophyte community to that of skin, and the compositions between the two tissue types were not significantly different ($p = 0.525$; Fig. 4). Similar to skin tissues, the IM treatment contained more unidentified taxa, and was dominated by *Penicillium*, while the O and U treatments contained high percentages of *Zygophiala* (34%) and *Stomiopeltis* (22%), respectively, which are both SBFS pathogens (Fig. 7). This is somewhat surprising, as the SBFS complex is not known to invade living plant cells, and could perhaps be a result of contamination that occurred when separating fruit tissues. However, it may also be possible that these pathogens could have extended into pulp tissues by expansion through intercellular spaces, especially given the high levels of infection observed on the fruit. Other taxa with high relative abundance in pulp tissues included *Alternaria* and *Colletotrichum* in the O treatment, *Trichothecium* and *Exophiala* in the IM treatment, and *Metschnikowia* and *Cladosporium* across treatments, the latter two of which were more abundant in pulp tissues than

skin (Fig. 7). However, in contrast to skin tissues, there were no differences in endophyte diversity metrics within pulp tissues (Fig. 5). Thus, overall, skin, and to some extent, pulp, endophyte composition and diversity seems to be driven by the relative abundances of SBFS species, with the highest percentage found in the U treatment, an intermediate amount in the O treatment, and the lowest amount in the IM treatment, leading to an increase in skin endophyte diversity in fruits receiving pesticide treatments, contrary to expectations.

Interestingly, seed richness was higher in the U treatment than the IM treatment ($p = 0.001$), with intermediate levels in the O treatment (Fig. 5A), a reversal of the pattern observed in skin diversity (Fig. 5C). This suggests that treatments may not only affect the surface-level endophytes, but may also cause shifts in interior communities as well. The dominant taxa in seeds were largely unidentified at the genus-level, and these taxa were particularly abundant in the IM treatment (55% relative abundance), but were also present at high levels in the O and U treatments (~23% relative abundance; Fig. 7). Thus, seeds, which not only held the highest proportion of unidentified taxa, but also contained the highest richness and diversity (all $p < 0.0381$) as well as an endophyte community distinct from those of other tissues (both $p = 0.002$; Fig. 4), represent a fascinating and relatively unexplored region of biotic interactions, and may in fact be a hotspot for fungal endophyte diversity. The importance of seeds has also been noted in other studies, as they have been found to contain the highest bacterial abundance in apple fruits (Wassermann et al. 2019b). However, the role of the seed microbiome in influencing plant traits remains largely unexplored, despite its potential importance, as seed endophytes may be vertically transmitted to future generations (Shade et al. 2017; Johnston-Monje and Raizada 2011; Truyens et al. 2015), allowing for the potential spread of endophytes along with seed dispersal (Barret et al. 2016; Nelson 2018). Indeed, these endophytes may provide a protective reservoir for a seed dispersed into an unfavorable environment; for example, cactus seed endophytes are capable of weathering rocks, allowing the cactus to establish in areas where no soil exists (Puente et al. 2009). Furthermore, the seed microbiome has also been shown to affect germination success (Nelson 2004) and seed dormancy (Goggin et al. 2015), traits which suggest the potential for agricultural applications (Gopal and Gupta 2016; Berg and Raaijmakers 2018). Thus, understanding the benefits conferred by an associated microbial consortium in seeds could be important for improving breeding strategies in certain crop species.

Phenolic Chemistry

Overall, the impact of treatment-established communities of biotic interactions on fruit phenolic chemistry varied by sampling season. In the mid-season sampling period, there were no treatment effects on phenolic composition, total phenolic concentrations, richness, evenness, or diversity (all $p > 0.149$; Fig. 8). This suggests that either the early season fruit chemical composition is driven largely by genetics and abiotic conditions, with minimal influence from biotic interactions, or that the biotic interactions impacting pesticide and untreated fruits were not sufficiently variable, and did not induce different levels of chemical defenses. Specifically, this lack of treatment effect could be due to overall lower fungal damage during the mid-season compared to the late-season (Fig. 1E-F), as well as reduced coverage of sooty blotch and flyspeck, which had not yet expanded to cover the entire fruit, creating the dramatic differences in fruit surface environments observed in the late-season (Fig. 2). Despite lower damage levels, however, mid-season skin tissues contained higher concentrations of phenolic compounds than in the late-season, although this pattern was reversed for pulp tissues (Fig. 16A). This increased skin phenolic concentrations is likely due primarily to developmental patterns of constitutive fruit chemistry, as phytochemical concentrations are frequently higher in unripe fruits, and decline during the ripening stages in order to increase fruit palatability for seed dispersers (Cipollini and Levey 1997). It may also be that the naturally heightened phenolic concentrations in unripe fruits resulted in lower levels of pathogen infection rates across treatments; in fact, some pathogens are known to remain relatively dormant on host surfaces until the stage of fruit ripening and corresponding decrease in phytochemical content, at which point they invade fruit tissues (Lattanzio et al. 2012). Alternatively, the increased skin phenolic concentrations may have also resulted from an environmental stressor, such as UV exposure. Phenolic compounds are produced in response to UV light; particularly flavonols (Kuhlmann and Mueller 2011), which were the dominant compound class within skin tissues (Fig. 10). Perhaps the low biotic pressures combined with the minimal coverage of SBFS, which may protect its host from UV damage by either physical coverage of fruit surfaces or by release of UV-defending chemicals such as melanin (Xu et al. 2017), masked treatment differences, and instead resulted in the upregulation of skin phenolics across treatments in response to higher mid-season UV exposure. Regardless, this difference in treatment effects between sampling seasons indicates a potential temporal variability in the relationship between biotic interactions and induced phytochemistry.

In contrast, there were treatment effects during the late-season, as IM and U phenolic compositions were distinct in skin tissues ($p = 0.05$), and I and M compositions were distinct in pulp tissues ($p < 0.03$; Fig. 9). Furthermore, the non-antimicrobial-treated fruits (I, U, and O) tended to display patterns of higher phenolic diversity than antimicrobial-treated, although this was variable, as effects among the three treatments were not consistent across diversity metrics or tissue type (Fig. 12). Thus, although there were some treatment effects, treatments did not produce distinct profiles of phenolic chemistry as expected. It is likely that distinctions among treatment groups were blurred due to a season with low insect pressures and high fungal damage; thus, treatments generally followed patterns falling into two broad categories: those that received antimicrobials (IM, M), and those that did not (I, U, and O). Interestingly, the dominant pathogens observed in the orchard, sooty blotch and flyspeck, do not necessarily induce a defensive response. However, we did observe damage from other fungal pathogens, and it was clear that overall tree health was noticeably affected by treatments, as trees treated with the U, O, and I treatments yielded lower fruit levels (personal observation) and produced smaller fruits, likely due to leaf damage and a subsequent reduction in photosynthetic rates (Teixeira et al. 2020). Therefore, the trend of higher pulp phenolic concentrations and diversity in I, U, and O treatments compared to M was likely caused by induced defenses from increased biotic damage levels, either from direct damage to the fruit or from systemic effects across the tree. Furthermore, in addition to low insect pressures that likely failed to separate the I treatment from the U and O treatments, the lack of fine-scale differences among non-antimicrobial treatments may also have been driven by the higher biotic variability experienced by these fruits, leading to inconsistent treatment effects; for example, a given U or O-treated fruit could have sustained fungal damage, insect damage, or both, resulting in substantially different chemical profiles. These multi-antagonist interactions may also have resulted in crosstalk among plant hormone pathways, potentially inhibiting the production of certain phytochemicals. This may explain why the I treatment, which was exposed only to fungal pathogens and was thus less likely to experience antagonistic crosstalk, sometimes showed a stronger induced response than O and U treatments (Fig. 8F; 12B,D; 13C-D). In contrast, the M and IM treatments were not subject to as much fruit-level variation. It is unclear why the IM treatment, which was expected to contain the lowest phenolic concentrations, instead sometimes displayed trends of higher phenolic chemistry than M, despite equivalent, or in some cases, reduced, damage levels (Fig. 2). Perhaps this was a

consequence of applying both pesticide types simultaneously; more likely, however, this was due to random fluctuations in fruit baseline chemistry. Nevertheless, although there were treatment effects in late-season fruits, fine-scale distinctions among treatment groups were masked by high fruit-level variability and low insect damage levels, preventing the expected pattern of distinct biotic and chemical profiles of each treatment. Instead, we observed trends displayed by two general groups: fruits treated with antimicrobials (IM and M) and fruits exposed to fungal damage (I, U, and O).

The increased fruit phenolic concentrations and richness observed in non-antimicrobial late-season fruits occurred only in pulp tissues. Pulp richness was consistently increased in I, U, and O treatments compared to M (all $p < 0.03$), and O and I treatments contained higher total phenolic concentrations than M and IM treatments (Fig. 12E-H). In contrast, total phenolic concentrations in skin samples were marginally higher in the IM treatment (Fig. 12A). However, both skin and pulp diversity were marginally higher in the I treatment compared to M (both $p < 0.08$). This difference in total phenolic concentrations between tissues may be a result of location-specific functions of phenolic defenses; in skin tissues, which are exposed to the surrounding environment, the production of phenolic compounds may be driven primarily in response to abiotic stressors such as UV irradiation. Treatments may have indirectly impacted UV exposure through differences in SBFS coverage; while SBFS completely blanketed fruit surfaces of late-season I, U, and O fruits, perhaps providing physical protection from UV rays or chemical protection through their melanin production, M and IM fruits were minimally covered by these fungi, and were thus more exposed to UV light. Furthermore, this trend of higher concentration in skins could have been a response to pesticides themselves, as phytochemicals may be produced in response to stress; in fact, anthocyanins have been shown to increase in fungicide-treated fruits (Nwankno et al. 2012). However, again, neither mechanism explains the difference in total phenolic concentrations between the M and IM treatments (Fig. 12A). Another consideration is that the IM treatment contained the highest fungal endophyte diversity (Fig. 5); perhaps these microbial taxa may be inducing a chemical response without increasing visible fruit damage levels. In contrast to skin tissues, phenolic production in pulp tissues may have been driven primarily by biotic interactions; as this tissue lacks the protective physical defenses found in skin, it may be especially vulnerable to damage. Perhaps, when fruits are attacked by biotic antagonists, plants respond by mobilizing phenolics to protect the pulp tissues, where

severe damage may be caused by interior-spreading rots or tunneling larvae. Thus, while the trend of increased phenolic content in IM skin tissues may be a response to higher UV exposure, the increased phenolic content in I, U, and O pulp tissues may be a response to the higher levels of biotic damage sustained in these treatments, either occurring directly on the fruit or resulting from a systemic priming effect throughout the highly-stressed tree, which was observed to drop fruit and sustain substantial leaf damage, potentially reducing photosynthetic capability. Therefore, it is possible that different functions of phenolic compounds may be driving opposing patterns of total concentrations in skin and pulp tissues across treatments. It should be noted, however, that these functions are not exclusive, and phenolic compounds may serve multiple functions simultaneously; skin flavonols, for example, both protect against UV light and defend against pests and pathogens (Kuhlmann and Mueller 2011). Thus, identifying the primary factor driving upregulation of phenolic compounds is a complex task in such variable conditions as field environments, and may vary locally across plant tissues.

However, although overall patterns of total phenolic concentrations varied by sampling season and tissue type, there were several compounds produced in higher concentrations in the non-antimicrobial treated fruits across tissue type and sampling season. Specifically, compound D, a benzoic acid, was upregulated in I, U, and O treatments in mid-season and late-season skin and pulp tissues, indicating a strong induced response to fungal damage (Fig. 13). Similarly, chlorogenic acid, a hydroxycinnamic acid, was upregulated in the I, U, and O treatments in late-season skin and pulp tissues (Fig. 14). Finally, compound C, compound H, and compound P, all benzoic acids, were upregulated in the I treatment in 'Golden Delicious' tissues (Fig. 15A-D). In contrast, there were some compounds upregulated in the M and IM treatments, such as procyanidin B2, a flavan-3-ol, and quercitrin, a flavonol (Fig. 15E-F). Again, the production of different compound classes among the antimicrobial and non-antimicrobial treatments may be a consequence of diverse functions of phenolic compounds. Flavonols and anthocyanins, which showed trends of higher concentrations in the IM and M late-season skin samples (Fig. 10;15E), were present almost exclusively in skin tissues (Fig. 10), and are known to protect against UV stress, consistent with their location within fruits (Downey et al. 2003; Smith and Markham 1998; Koes et al. 1994). They furthermore affect fruit pigmentation to attract seed dispersers, as anthocyanins are responsible for the red fruit color in apples, and flavonols may form complexes with anthocyanins to alter pigment (Koes et al. 1994). Therefore, there are a variety of biotic and

abiotic interactions that may influence flavonol and anthocyanin production, in addition to pest or pathogen damage. Pulp tissues contained higher percentages of benzoic acids and hydroxycinnamic acids than skin, and the relative abundance of these classes tended to increase in the O, U, and I treatments (Fig. 10). These compound classes were likely upregulated in response to biotic damage. There may also be synergistic effects among these compounds; chlorogenic acid, which was notably upregulated in response to fungal damage, has been shown to have enhanced efficacy when coupled with phloridzin, a dihydrochalcone (Lattanzio et al. 2012). While there appears to be a general pattern of fungal damage driving increased production of benzoic acids, hydroxycinnamic acids and dihydrochalcones in I, U, and O pulp tissues, and UV damage driving increased flavonol and anthocyanin production in IM and M treatments, these compound classes are not exclusively confined to these functions. Flavonols have defensive benefits, and hydroxycinnamic acids also reduce UV stress (Kuhlmann and Müller 2011). In short, while several individual compounds and compound classes appear to have been upregulated in response to fungal damage, it is likely that other compounds and compound classes were upregulated in response to UV stress, masking some of the overall trends in total phenolic concentrations by treatment group.

In addition to variation of treatment impacts on phenolic chemistry by tissue type and sampling season, treatment effects were also sometimes cultivar-dependent. ‘York’ fruits sustained higher damage levels than ‘Golden Delicious’ overall (Fig. 1), despite the higher total phenolic concentrations and richness in ‘York’ mid-season seed, late-season skin, and late-season pulp tissues (Figs. 8,12). Furthermore, these cultivars contained somewhat different compositions of phenolic compounds (Fig 11), and more compounds were significantly upregulated in ‘Golden Delicious’ fruits than in ‘York’ (Table 3). Thus, a compound that is strongly upregulated in response to a pathogen in one cultivar may not necessarily be produced in another, perhaps contributing to differences in cultivar susceptibility to biotic damage. Therefore, cultivar-level variation represents another layer of complexity in interpreting plant induced responses to biotic interactions.

Phenolic chemical diversity vs. fungal endophyte diversity

Finally, we compared phenolic diversity to fungal endophyte diversity within each fruit tissue sample to evaluate the relationship between phytochemical and endophyte diversity. There was a significant increase of pulp phenolic Shannon diversity with endophyte Shannon diversity ($p = 0.03$; $R^2 = 0.21$; Fig. 17A). There were also non-significant trends of a positive relationship for evenness, richness, and diversity across skin and pulp samples, although there was a negative trend for skin evenness (Fig. 17C), and many of these relationships were weak (all $p > 0.15$, all $R^2 < 0.12$; Fig. 17). These trends could potentially be strengthened by an enhanced sample size, as only a subset of samples was assessed for both fungal endophyte and phenolic diversity. Furthermore, to gain a more complete understanding of this relationship, there is a need to include a comparison of phenolic concentrations with endophyte absolute abundance, which could potentially display a stronger correlation than diversity metrics. Nevertheless, this positive relationship offers tantalizing directions for future investigations linking fruit metabolite and microbiome communities. It is unclear which variable is driving these positive trends; it could be that the presence of microbial taxa may induce a defensive response from the host plant, resulting in increased metabolite diversity. Indeed, endophyte colonization has been shown to increase host phytochemical production, even when the plant tissues are undamaged (Pineda et al. 2010). Furthermore, endophytes produce their own metabolites, including phenolic compounds (Pan et al. 2017), to mediate interactions with both the host plant and with other microorganisms, which could also increase the metabolite diversity within plant tissues. Alternatively, plants could attract or repel microbial taxa through phytochemical production, using a diverse set of metabolites to shape the community structure of the associated microbiome (Chagas et al. 2018). Overall, the relationship between the plant microbiome and plant chemistry is largely unexplored, despite its importance in understanding microbial community structure and plant defense allocations, as well as its potential for developing sustainable management strategies.

Conclusion

Induced fruit phenolic chemical responses to fungal damage were primarily observed in pulp tissues, although there were certain compounds that increased across tissue types and sampling periods. Low insect damage levels limited fine-scale distinctions among the five treatment groups, creating instead two broad categories that often shared general trends: antimicrobial-treated (M and IM) and non-antimicrobial treated (I, U, and O). Furthermore, the biotic communities appeared to be strongly affected by the presence of the SBFS complex, which reduced fungal endophyte diversity on skin surfaces and potentially altered fruit environments by providing protection against UV damage. Thus, there were potentially two opposing forces driving fruit phenolic production: UV-protecting flavonols and anthocyanins displayed trends of upregulation in antimicrobial-treated late-season skin tissues (IM and M treatments) that were not covered by SBFS pathogens and were exposed to pesticide regimes, while pulp phenolics such as chlorogenic acid, a hydroxycinnamic acid, and compound D, an unidentified benzoic acid, were upregulated in response to the higher fungal damage received in the late-season U, I and O treatments. Additionally, there was a positive relationship between fruit pulp phenolic diversity and fungal endophyte diversity, which has interesting implications for understanding endophyte community structure and localized plant metabolite production. In conclusion, the multi-functionality of phenolic compounds and the variability among sampling seasons, tissue types, cultivars, and baseline chemistry of individual fruits led to variation in patterns of fruit phenolic content and diversity that often limited our ability to detect a strong induced response to biotic pressures among treatments. Thus, to fully understand fruit responses to biotic interactions, defense chemistry needs to be considered in the context of environmental factors. Future work should explore the mechanism between endophyte and chemical diversity, as well as directly compare induced fruit chemical responses to distinct types of pests, pathogens, and microbial consortia in specific environmental contexts. Although the relationship between biotic interactions, both at the external pest and pathogen and internal microbiome level, and fruit chemistry is highly complex and variable, it remains an important area of investigation in understanding drivers of chemical diversity and microbiome community composition, both of which can have profound impacts on sustainable agriculture practices.

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CHAPTER 2: Impact of exogenous hormone application on fruit chemistry and resistance to pests and pathogens in apple fruits

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Abstract

There is a critical need to develop methods preventing crop losses to pests and pathogens while reducing reliance on unsustainable agricultural practices. The use of elicitors that activate a plant's natural defenses, such as jasmonic acid (JA), salicylic acid (SA), and melatonin (M), offers a promising avenue for sustainable pest control that is also potentially more flexible and health-promoting. However, much is still unknown about the feasibility of using these elicitors in a field environment, particularly when applied to fruit-producing perennials such as apples. There is also crosstalk that exists among the hormone pathways, which may create a tradeoff between tailoring defenses against a specific attacker and defending against concurrent attacks from multiple types of antagonists. Here, we apply melatonin (M), as well as synthetic analogues of JA (J) and SA (S) independently and simultaneously (JS) to apple fruits, and examine treatment effects on the concentration and diversity of defensive phenolic compounds. We further assess whether these treatments increase resistance against two classes of antagonists: a fungal pathogen (bitter rot; *Colletotrichum sp*.), and an insect pest (apple maggot; *Rhagoletis pomonella*), both independently and at the same time. We found no effects of treatments on phenolic chemistry, highlighting the sensitivity of these applications to factors such as dosage, timing, cultivar type, or environmental conditions. However, the JS treatment reduced insect pupal emergence, likely by decreasing oviposition rates, suggesting this treatment activated some type of non-phenolic defense. An improved understanding of the variation behind these hormones in inducing defensive responses as well as of the mechanism driving their effects on specific antagonists will prove fundamental for effectively utilizing these elicitors as part of a more sustainable and resilient pest management strategy.

Introduction

Modern agriculture faces the urgent challenge of feeding a growing population without inflicting high environmental costs (Ehrlich and Harte 2015). One crucial element of crop management in need of improvement is pest control, as 18 and 16% of major crop produce worldwide is lost from pest and pathogen damage, respectively (Oerke 2006). Pesticide use,

although initially effective, often results in subsequent rises in pest resistance (Palumbi 2001) and in environmental consequences such as the loss of stream biodiversity or pollinator decline (Beketov et al. 2013; Goulson et al. 2015). These cascading negative effects are driving a shift towards more sustainable pest control methods; one such avenue that has been proposed is the harnessing of plant natural defense systems, which could provide a more flexible and resilient solution to the adaptive responses of pests and pathogens as well as reduce reliance on environmentally unsustainable agricultural practices (Gozzo 2003).

Plants have evolved an arsenal of chemical defenses that allow them to respond dynamically to various types of attacks. These defensive compounds may be present in the plant tissue at all times as a constitutive defense, but may also be produced directly in response to an attack as an induced defense (Karban and Baldwin 2007). When a plant is damaged, it responds with the upregulation of phytochemical defenses and the priming of plant systems to respond more efficiently to future attack (Dangl and Jones 2001; Chisholm et al. 2006). These defenses may include a diverse mixture of chemical compounds that act as repellants, deterrents, or arrestants to the attacker (Bennett and Wallsgrove 1994), as well as the release of volatile compounds that attract predators or parasitoids of the plant antagonist (Kessler and Baldwin 2001). Furthermore, this response has been shown to be systemic, such that damage accrued in one region of the plant may lead to enhanced defenses throughout the entire plant, and even in neighboring plants (Karban and Baldwin 2007). The dynamic, multi-faceted nature of this defensive response provides an advantage over pesticide use, as it is more resilient against pest and pathogen counter-adaptations. Furthermore, many of these defense compounds have also been shown to provide enhanced fruit flavors, fruit quality, and human health benefits (Tomas-Barberan and Espin 2001), suggesting that, in some cases, a plant with higher phytochemical content could be simultaneously better-defended and of higher quality for human consumers. Thus, manipulating plant metabolism to induce these defenses has been an area of intense focus for developing novel agricultural management strategies (War et al. 2012).

Several methods have been developed to activate these plant defenses, including genetic modification (Birkett and Pickett 2014; Beale et al. 2006; Leckie et al. 2012; Jirschitzka et al. 2013) and the application of elicitors that induce a defensive response (Gozzo 2003; Agrawal 2011; Howe and Jander 2008; Sharma 2008). These elicitors include hormones that activate defense pathways to produce phytochemical end products; however, these pathways are complex

and may vary in effectiveness against different types of antagonists. For example, biotrophic pathogens and phloem-feeding insects such as aphids activate a plant defensive response through the salicylic acid (SA) pathway, while damage from leaf-chewing insects such as Lepidoptera and necrotrophic pathogens stimulate the jasmonic acid (JA) pathway (Glazebrook 2005). This has been suggested to be driven by the different feeding habits of these two classes, as a defense that functions in localized areas where pathogens or phloem-feeding aphids feed may not be as effective against highly mobile, leaf-chewing herbivores (Kessler and Baldwin 2002).

The exogenous application of these defense hormones has been shown to be successful in a wide variety of crop systems. For example, the application of salicylic acid has been shown to reduce damage from the fungal pathogens *Venturia inaequalis*, *Guignardia bidwellii* and *Penicillium expansum* in apples (Abbasi et al. 2016; da Rocha Neto and Di Piero 2013), *Meloidogyne incognita* and *Fusarium oxysporum* in tomatoes (Nandi et al. 2000; Mandal et al. 2009), and the bacteria *Erwinia amylovora* in pears (Ghahremani and Abdollahi 2011). Jasmonic acid has been shown to reduce damage from the beet armyworm (*Spodoptera exigua*) in tomato plants (Thaler et al. 1996), defend against grasshopper nymphs (*Trimerotropis pallidipennis*) in tobacco (Baldwin 1998), and protect against a weevil (*Lissorhoptrus oryzophilus*) in rice (Hamm et al. 2010). This defensive success is driven by the production of phytochemicals, as both JA and SA applications have been shown to increase certain classes of metabolites, including volatiles (Dicke et al. 1999; Smart et al. 2013), glucosinolates (Brader et al. 2001), and total phenolics (Taipalensuu et al. 1997). Furthermore, untargeted metabolic fingerprinting has revealed dramatic changes within the overall phytometabolome after treatment application, and has also shown that SA and JA produce distinct metabolic profiles (Sutter and Müller 2011).

However, these two defense pathways may not always occur effectively at the same time, as crosstalk has been shown to exist between them (Glazebrook 2005). This crosstalk is prevalent among many plant taxa, and typically takes the form of a reciprocal antagonism in which SA inhibits the production of JA (Thaler et al. 2012; Leon-Reyes et al. 2010); however, there have been instances where JA can inhibit SA (Rayapuram and Baldwin 2007), and where the JA and SA pathways can act in a synergistic manner (Schenk et al. 2000; Devadas et al. 2002). It has been hypothesized that this crosstalk serves as a cost-saving adaptive response of the plant that allows for the tailoring of defenses against specific antagonists (i.e., the Adaptive Tailoring Hypothesis); however, the extent to which crosstalk is a beneficial adaptation is

debated (Thaler et al. 2012). This antagonism can be exploited by pests and pathogens, allowing the antagonist to inhibit the tailored defense by promoting the less effective one (Cui et al. 2005; Kloth et al. 2016). For example, the saliva of the beet armyworm caterpillar (*Spodoptera exigua*) induces the SA pathway, inhibiting the JA pathway, which is the preferred defense against chewing insect herbivores (Weech et al. 2008). In short, the nature of this crosstalk is complex and variable, and much is still unknown regarding its adaptive function. Therefore, in order for hormone-induced defenses to be a viable management strategy, there is a need for improved understanding of the consequences arising from these interacting pathways for overall plant defensive ability.

Although it has been well-demonstrated that both JA and SA are effective defense activators when applied separately, less is known about the effects of simultaneous application as a management strategy, in terms of their effects on both plant chemistry and resistance ability. However, it has been shown that the application of SA and JA, both separately and together, produced distinct metabolic profiles in *Plantago lanceolata*. Specifically, the treatments of JA and SA alone resulted in the synthesis of JA and SA-specific metabolite targets, and the simultaneous application of these hormones resulted in the production of some, but not all, of these specific targets, likely due to the antagonistic crosstalk between the two pathways. The combined JA-SA treatment also produced common targets, which are metabolites that are regulated independently by each hormone, and interaction targets, which are metabolites required by the presence of both hormones (Schweiger et al. 2014). This suggests that, although JA and SA individually may be better-suited for defense against specific targets, the simultaneous application of these hormones could produce an overall higher level of chemical diversity, and thus provide an enhanced defense against concurrent attacks from multiple types of antagonists. However, while it has been shown that the simultaneous application of hormones was less effective against herbivory than individual treatments (Schweiger et al. 2014; Cipollini et al. 2004), the impact of simultaneous applications against concurrent attacks from multiple types of antagonists has not been well-investigated. Thus, it remains unknown if the application of SA and JA in concert could provide an advantage to crops facing multiple-antagonist attack, albeit at the expense of a more effective tailored defense against an individual target.

Finally, there are other hormones that have been suggested to act as plant defense activators, such as melatonin, which was only recently discovered in plants (Dubbels et al. 1995;

Hattori et al. 1995), and thus its metabolic functions are still being deciphered. However, it has been suggested to play a key role in plant immune response (Arnao and Hernández-Ruiz 2018), working upstream of the SA and JA signaling pathways by inducing their biosynthesis in an interaction with nitric oxide (Lee and Back 2016; Lee et al. 2014; Shi et al. 2015; Arnao and Hernández-Ruiz 2006), although its exact role in modulating defense responses remains unclear (Arnao and Hernández-Ruiz 2006). Furthermore, the application of melatonin has been shown to protect plants against abiotic stresses (Li et al. 2016) as well as fungal pathogens (Yin et al. 2013; Okatan et al. 2018), and has also been shown to increase phenolic content in apples (Okatan et al. 2018). As melatonin is a relatively recent discovery in plants, its impacts on crop chemistry and defense induction are not well understood. To our knowledge, no studies have compared the effects of the exogenous application of melatonin, SA, and JA on plant chemistry and resistance to antagonists in an agricultural environment.

To assess these defense-activating hormones as a management strategy for an important crop species, we applied exogenous treatments of 1) a commercial product containing acibenzolar-S-methyl (S), a synthetic analogue of SA, 2) a commercial product containing prohydrojasmon (J), an analogue of JA, 3) a combined treatment of S and J (JS), 4) melatonin (M), and 5) a water control (C), to apple fruits to assess their effectiveness as defense activators. We had three specific objectives: 1) to test how different exogenous hormone applications, applied both independently and concurrently, impact fruit phenolic chemistry and sugar content, 2) to examine how fruit phenolic chemistry changes over time following exogenous hormone application, and 3) to test how exogenous hormone application impacts resistance to single and combined pest pressure by measuring resistance to a fungal pathogen (*Colletotrichum sp)*, an insect pest (*Rhagoletis pomonella)*, and to both antagonists at the same time. We expected that all hormone treatments would induce a plant defensive response, resulting in higher phenolic concentrations and diversity than the control treatment (Objective 1), with a peak phenolic induction occurring in each of the treatments approximately 3-5 days after application, in line with other studies (Schweiger et al. 2014; Ziadi et al. 2001; Thiruvengadam et al. 2015; Cipollini and Sipe 2001), while untreated fruits would maintain a relatively constant level of phenolic chemistry over time (Objective 2). Furthermore, we expected the S and J treatments would produce distinctly different profiles of target phenolic compounds, in line with the Adaptive Tailoring Hypothesis (Thaler et al. 2012), such that the J treatment would produce metabolites

that provide the best defense against the insect pest, and the S treatment would produce metabolites that defend best against the fungal pathogen. We expected the JS treatment to have the highest overall phytochemical diversity, which would offer the best defense against multi-species attack, at the expense of effectiveness against each attacker individually, due to the antagonistic crosstalk between the hormone pathways. Finally, as melatonin is suggested to act upstream of both JA and SA biosynthesis, it was unclear how its chemical profile would compare to the other treatments; however, we expected it may most closely resemble the effects of the S application, due to its success in defending against fungal pathogens (Yin et al. 2013; Okatan et al. 2018). A conceptual model of predicted treatment effects on resistance to antagonists (Objective 3) is presented in Figure 1. Overall, the goals of this project were to provide insight into the use of these hormones as a defense management strategy by examining the potential trade-off between target specificity and effectiveness against multi-species attack, and elucidate the effects of treatments on apple fruit chemistry and resistance, informing the development of sustainable management strategies that may simultaneously improve crop defense and quality.

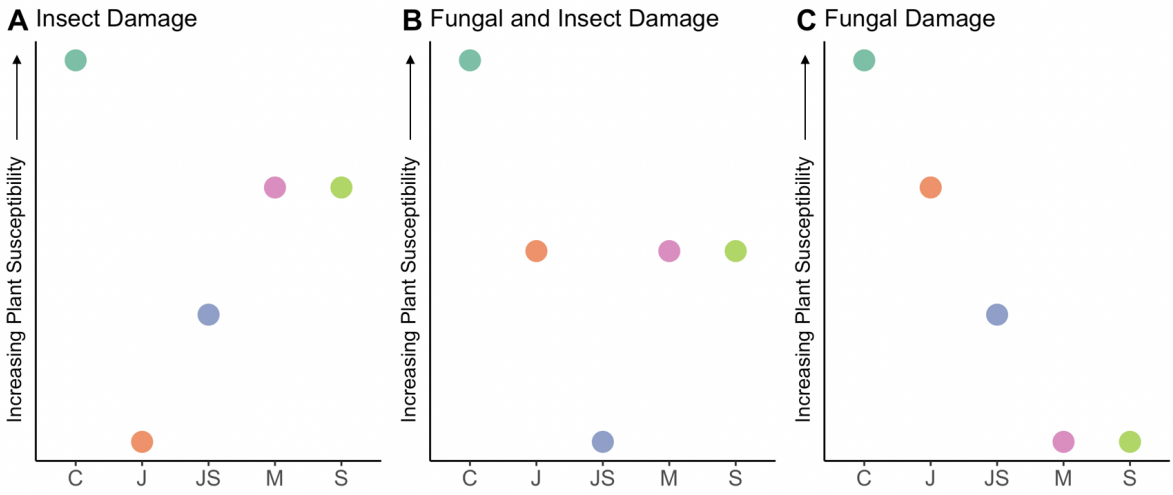

Figure 1. Conceptual model of fruit susceptibility to A) insect damage, B) combined fungal and insect damage, and C) fungal damage alone after applications of control (C), jasmonic acid analogue (J), combined jasmonic acid and salicylic acid analogues (JS), salicylic acid analogue (S) and melatonin (M) treatments. We predicted J-treated fruits would be least susceptible to insect damage (A), and M and S-treated fruits to be least susceptible to fungal damage (C), under the general assumption that insects are primarily affected by JA-dependent responses and

pathogens by SA-dependent defenses. Furthermore, we expected the JS treatment to defend best against both fungal and insect damage (B), at the expense of specificity to insects and fungi (A, C), in line with the Adaptive Tailoring Hypothesis.

Methods

Study System

Apples (*Malus pumila*) are an important agricultural product, with an annual wholesale value of $4 billion (USDA 2017; US Apple 2018), but the fruit is highly susceptible to pest and pathogen damage (Beers et al. 2003; Grove et al. 2003). Although apples produce defensive phenolic compounds (Lee et al. 2011; Cuthbertson et al. 2012), the domesticated cultivars contain lower concentrations and diversity than wild species, likely due to selection for fruit size at the expense of defense (Whitehead and Poveda 2019). Therefore, restoring the levels of these phytochemicals that have been lost over generations of selective breeding could prove an effective pest management strategy. Furthermore, as a long-lived perennial tree, apples represent a complex system for studying induced defenses, as it has been suggested that defense allocations may vary due to factors such as lifespan, growth rate, or habit (Cipollini et al. 2017; Karban and Baldwin 2007). Prior work on elicitor applications has focused primarily on annual, herbaceous crops, and has also frequently occurred under laboratory conditions or as a post-harvest treatment. Applying these hormones in a field experiment with apples, therefore, may provide new insights into their impacts on plant chemical responses and defensive ability.

Apple fruit tissue contains phenolic compounds, which are a diverse class of phytochemicals that function as a plant defensive response, and are found across a wide range of plant taxa (Lattanzio et al. 2006; Lindroth and Peterson 1988). Additionally, they have been shown to provide human health benefits, such as lowering blood pressure and cholesterol, inhibiting tumor growth, increasing antioxidant activity, and protecting against cancer and cardiovascular disease (Watanabe et al. 2006; Boyer and Liu 2004; Deschner et al. 1991; Guardia et al. 2001), making their accumulation in fruit tissue a desirable goal. Their production has been shown to be induced by the application of elicitors, as both JA and SA have caused an increase in total phenolic content (Bali et al. 2019; Thiruvengadam et al. 2015) and in the concentrations of specific compounds. For instance, in vegetative tissues, JA has been shown to cause an increase in chlorogenic acid, epicatechin, catechin, rutin, and anthocyanins (Cirak et al.

2020; Thiruvengadam et al. 2015), while SA has been shown to increase syringic acid, chlorogenic acid, caffeic acid, gentisic acid, p-coumaric acid, ferulic acid, and anthocyanins (Thiruvengadam et al. 2015; Kováčik et al. 2008).

Two prominent antagonists that attack apples in eastern North American orchards are the apple maggot (*Rhagoletis pomonella*) and bitter rot (*Colletotrichum sp)*. The apple maggot adult oviposits onto the apple fruit, where larvae emerge and feed internally on the pulp, often producing such substantial damage that the fruit falls to the ground, where the maggot emerges and pupates (Glass 1966). Bitter rot, which can be caused by several species of the ascomycete genus *Colletotrichum* (Munir et al. 2016), overwinters in wood or old fruit, releasing spores that infect fruit tissue and form a lesion that expands until the rot may cover the entirety of the fruit and descend into the pulp (Ellis 2008). These species were selected to represent two classes of antagonists that are affected by distinct defense pathways, as we expect that the apple maggot, a chewing insect in its larval stage, would be most affected by the JA pathway, and that bitter rot, a hemibiotrophic fungal pathogen, would be most affected by the SA pathway.

Treatment Application

The project took place at the Virginia Polytechnic Institute and State University's (Virginia Tech) Kentland Farm, which contains a 16-year old apple orchard composed of 'York' and 'Golden Delicious' cultivars grafted on M.26 rootstock apples. The orchard has historically been managed with conventional spray programs; however, during the season in which the project was conducted, trees only received early season fungicides and antibiotics through bloom in early May. In late June 2019, 59 Golden Delicious trees received one of the following 5 treatments, for a total of 11 -12 trees per treatment: 1) Blush2X® (Fine Americas, Inc.), a commercial product that contains prohydrojasmon, a JA analogue (J), 2) Actigard® 50WG (Syngenta, Inc.), a commercial product that contains acibenzolar-S-methyl, an SA analogue (S), 3) a combined application of S and J (JS), 4) melatonin (M; Sigma-Aldrich, CAT: M5250-250MG), and 5) a control of distilled water (C).

The J treatment consisted of Blush2X® (Fine Americas, Inc.), which is a commercial product that contains the active ingredient prohydrojasmon, an analogue of JA, and that was designed to enhance the color of fruits by stimulating the production of anthocyanins. Prior work has shown that prohydrojasmon has been successfully used to increase fruit color (Cetinbas et al.

2018), total phenolics and anthocyanin levels (Azis et al. 2019), as well as volatile emissions (Uefune et al. 2014). Preliminary work with Blush® in apples has also shown that whole-tree application across the growing season caused a reduction of damage from internal Lepidoptera, tarnished plant bug, and San Jose scale (Whitehead and Poveda, unpublished). Here, we applied Blush2X® at a concentration of 480 ppm in distilled water, as recommended by the supplier (personal conversation, Fine Americas, Inc.).

The S treatment, Actigard® 50WG (Syngenta, Inc.), is a commercial fungicide containing acibenzolar-S-methyl, an analogue of SA that has been shown to defend against bitter rot in Royal Gala apples (Alamino et al. 2013), induce defensive genes and decrease fire blight damage in Golden Delicious apples (Ziadi et al. 2001; Brisset et al. 2000), and to have an antagonistic crosstalk with JA, similar to that of SA (Lin et al. 2008; Doherty et al. 1988). Furthermore, application has been shown to increase the production of phenolic compounds in such systems as peas (Barilli et al. 2010) and muskmelon fruit (Zhang et al. 2011). It was applied at a concentration of 400 mg per L of distilled water, as used effectively in apples by Dugé de Bernonville et al. (2014).

The combined treatment of J and S (JS) in distilled water maintained the same concentrations as used in the separate treatments. Melatonin (M; Sigma-Aldrich ≥98%) was applied at a concentration of 0.5 mM in distilled water, similar to levels used in other studies with apples (Okatan et al. 2018; Li et al. 2016; Yin et al. 2013). The control treatment (C) consisted of distilled water.

Treatments were applied directly to the fruit using a hand sprayer, and fruits were sprayed until the liquid ran off of the fruit (i.e., full saturation). A total of eight fruits were sprayed in different parts of the tree: three for phenolic analysis, three for bioassays, and two backups in case of fruit loss. After five days, which was expected to be the approximated peak induction of induced phytochemical production based on prior studies (Schweiger et al. 2014; Ziadi et al. 2001; Thiruvengadam et al. 2015; Cipollini and Sipe 2001), three treated fruits were harvested per tree, and skin and pulp tissue samples were immediately collected by removing the fruit skin with a blade and collecting pulp tissue with a cork borer. Equal amounts of each of the three fruits were pooled to form a composite pulp and skin sample for each tree, in order to account for between-fruit variation within each tree. Samples were immediately placed in liquid nitrogen in the field and stored at -80°C until extracted. At the same time as tissue sample

collection, we measured the soluble solids content (SSC) of the fruit juice for each of the three fruits using a handheld refractometer BZ-20 (Veegee Analytical Instruments, Kirkland, USA) in order to evaluate fruit sugar content, a potentially important factor in impacting fruit resistance to fungal and insect attack. SSC values were averaged for each tree. Finally, three additional treated apples were harvested from each tree and stored at 4.5°C until used for bioassays (2-5 days).

Additionally, four trees of cv. 'Yellow Transparent' were selected to examine the change in phenolic content over time after the application of treatments, in order to identify the timing of a peak phenolic response. Each tree was assigned a different treatment (M, J, S, and JS), and seven fruits were sprayed with the designated treatment for each tree. A control and a treated apple were harvested from each tree on 1, 2, 3, 4, 5, 8, and 10 days after treatment applications, and a skin and pulp tissue sample were also collected for each fruit to determine the total phenolic content.

Pest and Pathogen Assays

For bioassays, three treatment groups of apples were established: insect exposure only, fungal exposure only, and simultaneous exposure to the insect and fungus. Apples selected for fungal exposure were wound-inoculated twice, once on each side of the fruit, with spores produced by a bitter rot culture (*Colletotrichum sp*.) supplied by Keith Yoder (Virginia Tech Agricultural Research and Extension Center, Winchester) that was grown on potato dextrose agar. All apples were placed in individual 32 oz. deli cups, and were elevated above sand using plastic tripod pizza box stackers, allowing for larval emergence from the fruit and subsequent burrowing into the sand for pupation (Fig. 1). All containers were placed in a growth chamber at the following settings: light 6AM to 10PM, 25℃, and dark 10PM to 6AM, 22℃, at 35% humidity. Apple maggots (*Rhagoletis pomonella*) were supplied from a colony maintained by Tracy Leskey (USDA-ARS, Kearneysville, WV). An adult male and female pair were placed in the container for mating and oviposition, and were removed after 11 days. The only treatments included in insect and combined insect-fungus bioassays were J, JS, S, and C; melatonin was excluded due to insufficient numbers of male flies.

After 11 days, all pairs of apple maggots were removed, and the width of fungal lesions were measured. A rating scale was also applied to assess the extent of lesion growth, in which each side of the apple that contained a lesion was assigned a value from 1 to 5, where 1 = <5%, 2 = 5-25%, 3 = 25-50%, 4 = 50-75%, and 5 = >75%. The mean value for lesion width and lesion rating scale was then taken for each fruit. Lesions were measured again 18 days after inoculation.

After pupal emergence, the number of pupae was recorded, and the mass of each pupa was measured (mg) to determine the mean pupal mass for each fruit.

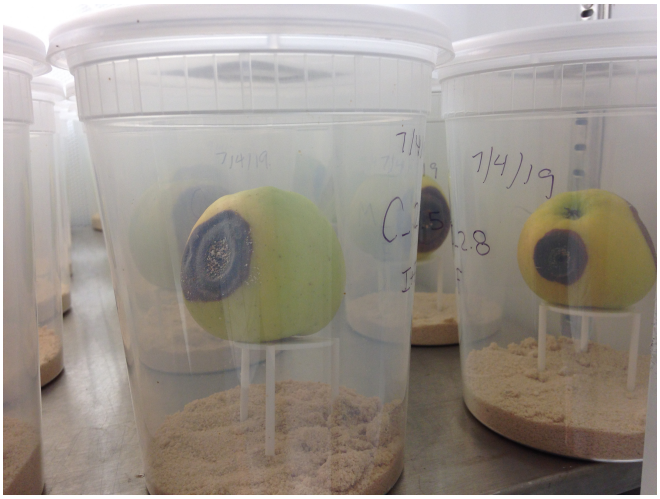

Figure 2. Photograph of apple bioassay containers inside the growth chamber. Fruits were exposed to three types of treatments: fungal inoculation of *Colletotrichum sp.,* exposure to adult pairs of the insect pest *Rhagoletis pomonella* for oviposition, or both. All fruits were stored in individual containers and elevated above a sand-covered floor to allow for larval emergence and pupation.

Phenolic Extractions

For analysis of phenolic content, we performed extractions on lyophilized fruit tissue samples using a solution of 70% methanol and 2% formic acid followed by high-performance liquid chromatography (HPLC), following the same procedures used in Chapter 1.

Fruit tissue samples that were collected to assess temporal variation in phenolic content after treatment application were analyzed only for total phenolic content using the Folin-Ciocalteu reagent (Ainsworth and Gillespie 2007). First, lyophilized, ground samples were extracted in 80% methanol, sonicated at room temperature for 20 min, and centrifuged for 5 min at 12000 rpm. The supernatant was then collected, and a second extraction was performed on the same plant material for a total of two extractions. The dilute Folin-Ciocalteu reagent (10% vol/vol) and sodium carbonate (Na_2CO_3) were added to each sample, and samples incubated for 2 hours prior to reading absorbance on a 96-well plate at 765 nm. Total phenolic concentrations were estimated by comparing values to a serial dilution of a gallic acid standard (gallic acid equivalents mg^{-1}).

Statistical Analyses

In order to determine how exogenous hormone application impacts fruit phenolic chemistry and sugar content (Objective 1), we calculated total phenolic concentrations, and used the R package vegan (Oksanen et al. 2019) to evaluate the following diversity metrics: richness, defined as the number of phenolic compounds in each sample, Pileou's evenness, which describes how equally abundant these compounds are within samples, and Shannon Diversity Index (H), a measure of both evenness and richness. Differences among treatment groups were evaluated using a linear model with treatment as the predictor variable and total phenolic concentrations or diversity metrics as the response variable. Each treatment was then compared to the control based on estimates of model coefficients in the linear model. If distributions were non-normal, values were first log-transformed. To identify specific individual compounds that varied among treatment groups, we used random forest classification models (Ranganathan and Borges 2010) conducted separately for skin and pulp tissues. We selected variables important for distinguishing among groups using the packages randomForest (Cutler and Wiener 2018) and Boruta (Kursa and Rudnicki 2018). We also classified compounds into six dominant phenolic compound classes in apples using UV spectra: flavan-3-ols, flavonols, anthocyanins, hydroxycinnamic acids, benzoic acids, and dihydrochalcones, and assessed treatment effects on total concentrations of these compound classes using a linear model, as described above. Finally, treatment effects on the soluble solids content (SSC) of fruit juice were also evaluated with a linear model.

To identify a potential peak induction after each treatment application (Objective 2), total phenolics time series were plotted using a nonlinear fitting function that contained a quadratic term, with the following formula: $y = ax+cx^2+b$, where a represents the slope coefficient, c is the coefficient of the quadratic term, and b is the intercept. This model was chosen due to the expectation that the application of treatments would produce either a single peak of highest total phenolic concentrations on a certain day (where $a > 0$ and $c < 0$) or a steady increase in phenolics over time (where $a > 0$ and c approximates 0); this equation provides the flexibility to model both patterns. Each of the four hormone treatments was assessed independently for treated and control fruits, and skin and pulp tissues were also assessed separately.

To assess fruit resistance to individual and combined pest and pathogen damage (Objective 3), we created a standardized metric of relative fruit susceptibility to damage. This was achieved for insect damage by dividing pupal weight values by the maximum value recorded, such that all values ranged from 0, indicating that no pupae emerged, to 1, indicating the highest possible value for pupal weight. This was repeated to assess susceptibility to the fungal pathogen by dividing fungal lesion width values measured at 11 days by their maximum value, creating a scale ranging from 0 to 1. These metrics were then averaged for fruits that were exposed to both types of antagonist. Differences among hormone treatments were assessed using a linear model.

Furthermore, pupal emergence and pupal weight were also analyzed separately for fruits exposed only to insects. Pupal emergence values were designated as 0 (no emergence) or 1 (at least one pupa emerged), and treatment differences were analyzed using a generalized linear model with a binomial distribution. Analysis of pupal weight only included pupae that emerged from the apple (i.e., all zero values were excluded), and differences were analyzed with a linear model with treatment as the predictor variable and pupal weight or emergence as the response variable.

All statistical analyses were performed in R v3.6.1 (R Core Team 2020). All figures were produced using the packages ggplot2 (Wickham et al. 2019), cowplot (Wilke 2019), gridExtra (Auguie and Antonov 2017), and ggsignif (Ahlmann-Eltze 2019).

Results

Fruit Phenolic Chemistry

Overall, there were no significant differences among treatments in total phenolic concentrations (pulp: $p = 0.61$, skin: $p = 0.98$), richness (pulp: $p = 0.47$, skin: $p = 0.22$), evenness (pulp: $p = 0.71$, skin: $p = 0.34$) or Shannon Diversity (pulp: $p = 0.84$, skin: $p = 0.38$), for pulp or skin samples. However, skin samples contained marginally higher diversity in the JS treatment ($p = 0.07$) than the control (Fig. 3A), and the S treatment displayed marginally lower levels of skin phenolic richness than the control treatment ($p = 0.05$), but higher evenness ($p = 0.07$).

We examined the ratio of phenolic concentrations between pulp and skin samples to evaluate the potential for a shift in defense allocation; that is, treatment applications could be causing a shift in the production of phenolic compounds from the skin tissues to the pulp. This was assessed by examining the ratio of phenolic content in pulp compared to skin for each treatment; however, there were found to be no differences among treatments in the ratio ($p = 0.94$; Fig. 4).

When examining treatments for variation in individual compounds, the random forest analysis revealed no important compounds that distinguished among treatments for either skin or pulp tissues (all mean importance <1.7 and all rejected in Boruta analysis). Furthermore, there were also no differences in total concentrations of compound classes among treatments (all $p > 0.31$; Fig.5; Fig. 6).

Finally, fruit sugar content was assessed by measuring the Soluble Solids Content (SSC) in the fruit juice. Similar to treatment effects on phenolic chemistry, there were no differences in SSC between treatments and the control ($p = 0.20$; Fig. 7).

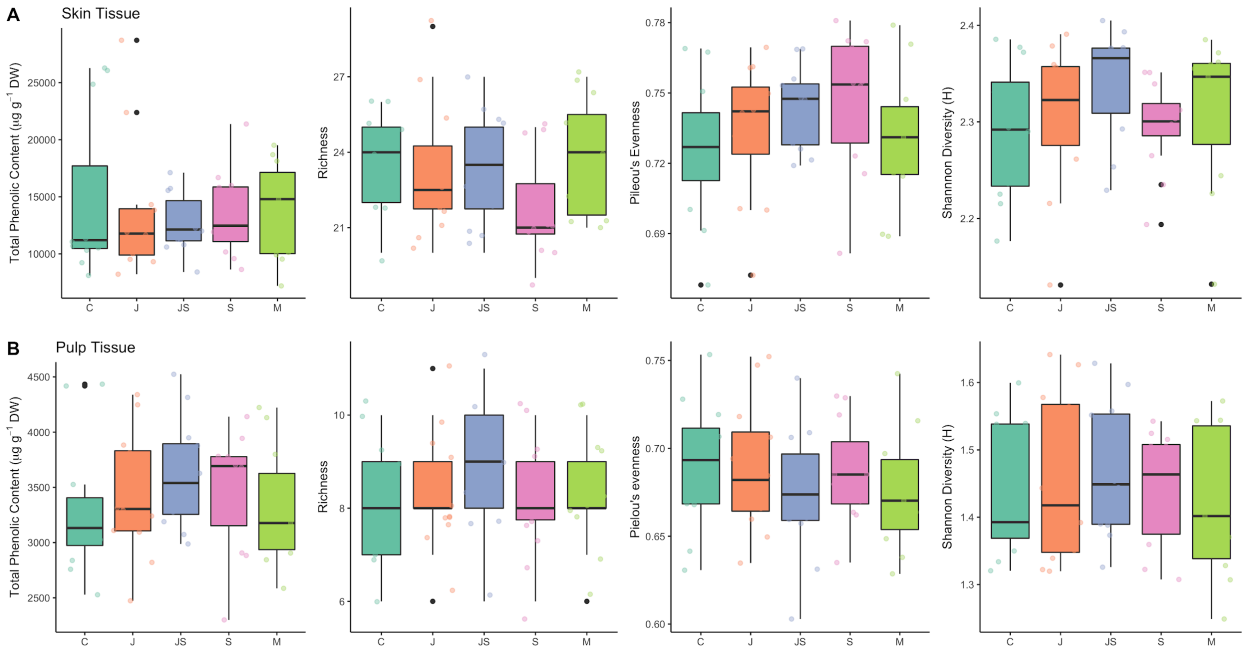

Figure 3. Total phenolic content (µg g^{-1} DW), richness, Pielou's evenness, and Shannon diversity index for A) skin tissue and B) pulp tissue samples after applications of control (C), jasmonic acid analogue (J), combined jasmonic acid and salicylic acid analogues (JS), salicylic acid analogue (S) and melatonin (M) treatments.

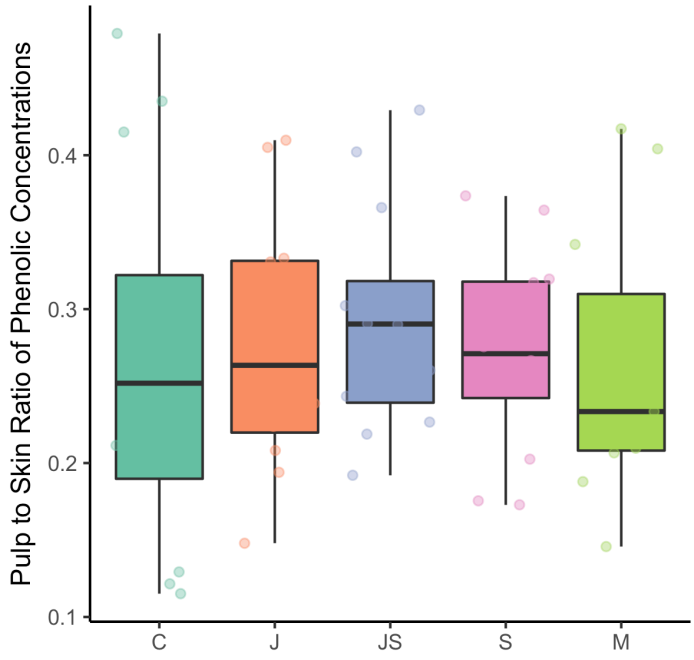

Figure 4. Ratio of total phenolic concentrations (μg g^{-1} DW) found in pulp tissue compared to skin tissue after applications of control (C), jasmonic acid analogue (J), combined jasmonic acid and salicylic acid analogues (JS), salicylic acid analogue (S) and melatonin (M) treatments.

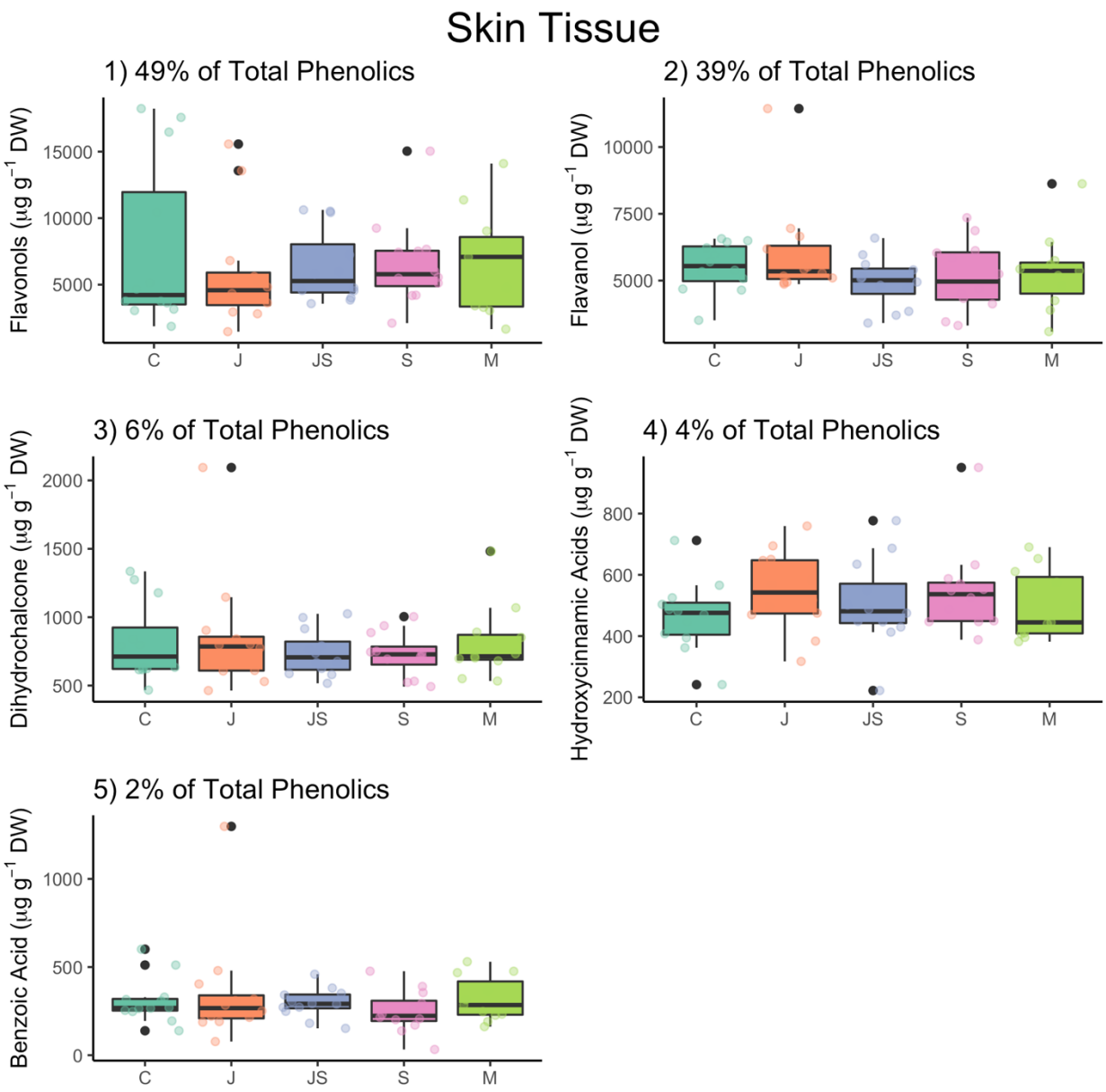

Figure 5. Total phenolic content (µg g^{-1} DW) by compound class in skin samples after applications of control (C), jasmonic acid analogue (J), combined jasmonic acid and salicylic acid analogues (JS), salicylic acid analogue (S) and melatonin (M) treatments. Classes are ranked from highest percentage of total phenolic composition (%) in skin tissue.

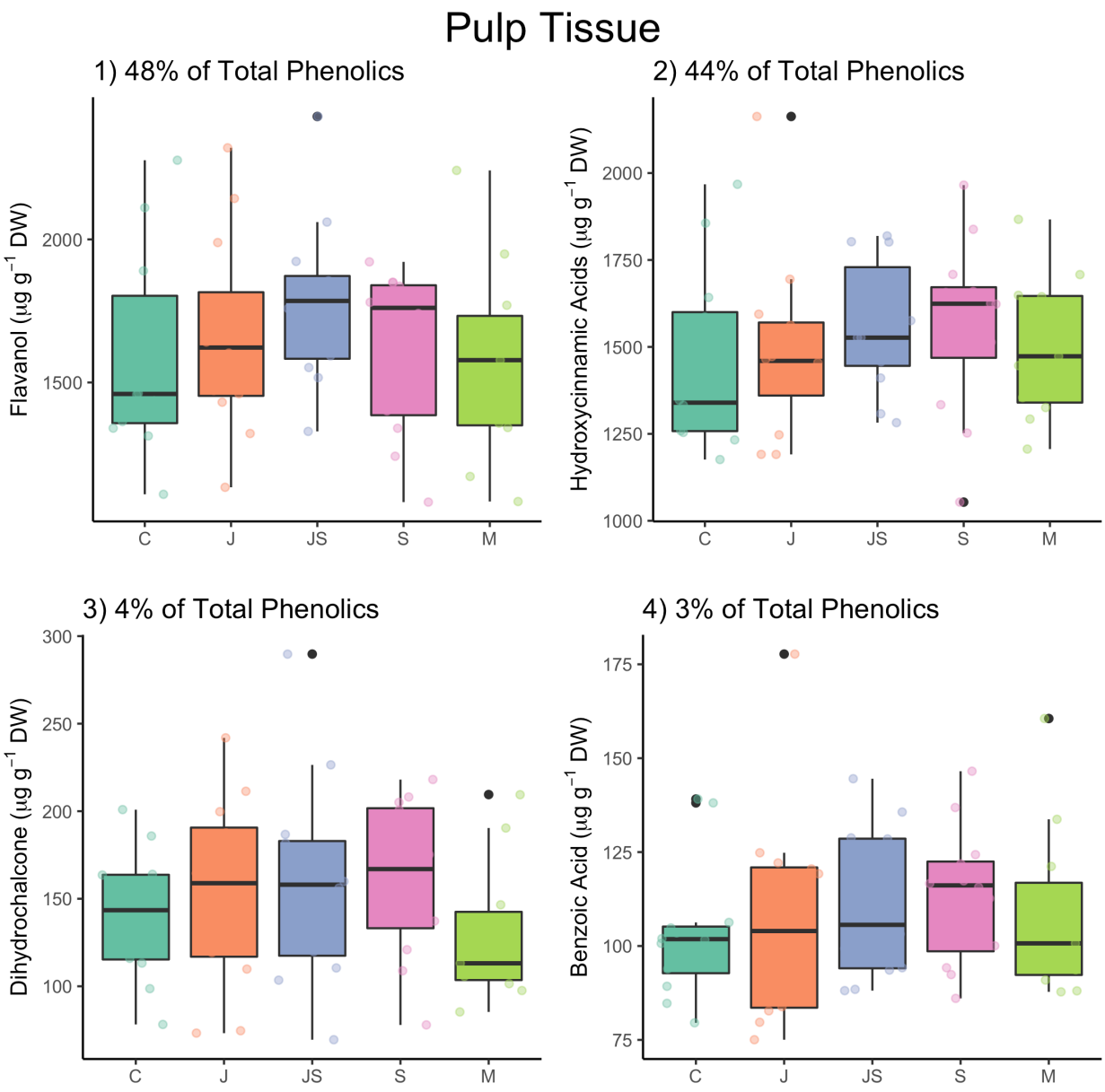

Figure 6. Total phenolic content (µg g^{-1} DW) by compound class in pulp samples after applications of control (C), jasmonic acid analogue (J), combined jasmonic acid and salicylic acid analogues (JS), salicylic acid analogue (S) and melatonin (M) treatments. Classes are ranked from highest percentage of total phenolic composition (%) in pulp tissue.

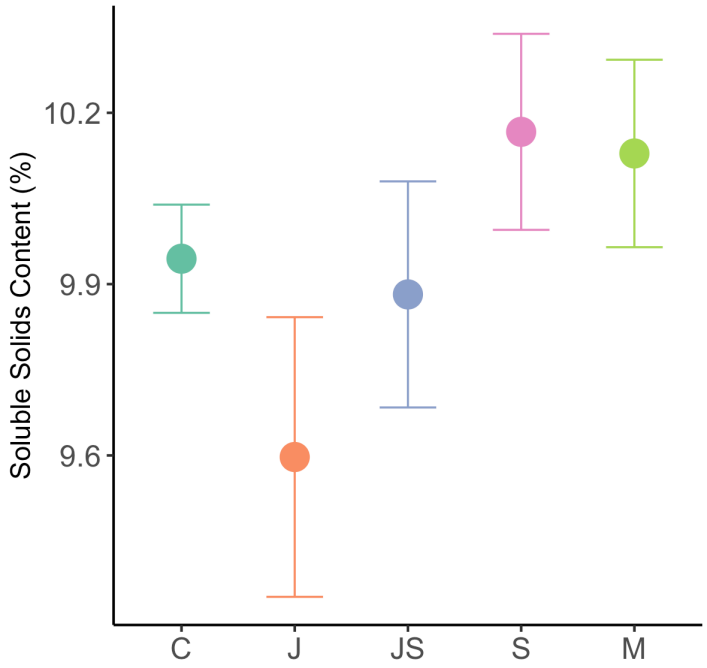

Figure 7. Soluble Solids Content (SSC) in apple fruit juice after applications of control (C), jasmonic acid analogue (J), combined jasmonic acid and salicylic acid analogues (JS), salicylic acid analogue (S) and melatonin (M) treatments. Bars indicate standard error.

Temporal Change in Total Phenolics

A peak induction could not be detected after application of any of the elicitors, as there were high levels of daily variation in phenolic chemistry of both treated and untreated fruits (Fig. 8). A nonlinear fitting function with a quadratic term revealed some trends, but they were largely non-significant and variable by treatment (Fig. 8). Ultimately, only skin tissues within the JS and S treatments displayed a temporal pattern, as JS-treated skin samples experienced an initial decline in phenolic content over time, as indicated by a negative value of the coefficient *a*, followed by an increase, as indicated by a positive value of the coefficient *c* (both $p = 0.01$), and the skin phenolic content of S-treated fruits also marginally declined over time ($a = -0.002$, $p = 0.06$; Fig. 8). The control fruits did not remain at a constant baseline level over time, as expected, and instead were subject to as much, or sometimes more, fluctuation than treated fruits in the same treatment tree (Fig. 8), although coefficients were not significant (all $p > 0.26$).

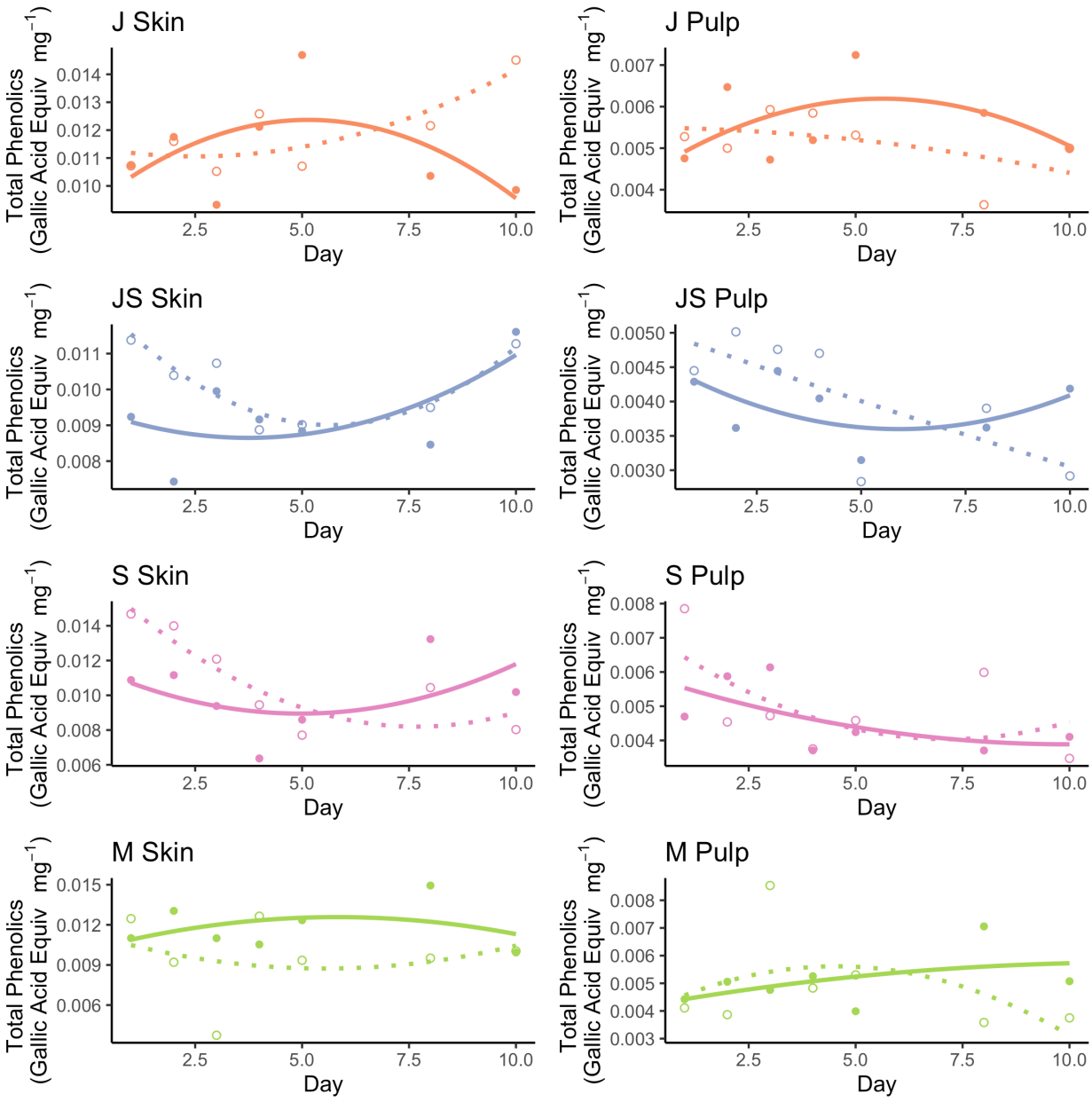

Figure 8. Total phenolic concentrations in hormone-treated apples (solid line, T) compared to untreated controls (dotted line, C) after application of jasmonic acid analogue (J), combined jasmonic acid and salicylic acid analogues (JS), salicylic acid analogue (S) and melatonin (M) treatments. Points represent individual fruits, and were fit with a nonlinear regression containing a quadratic term, with the formula: $y = ax+cx^2+b$.

Pest and Pathogen Assays

As shown in the conceptual model (Fig. 1), we expected J-treated apples to be least vulnerable to insect damage, S-treated to be least vulnerable to fungal damage, and JS-treated to be least vulnerable to multiple-species attack. Surprisingly, J-treated fruits performed poorly in terms of insect resistance, and S did not outperform JS in resistance to fungal damage (Fig. 9); however, there did appear to be some benefit of the JS and S treatments in comparison to the control, particularly in the insect assays (Fig. 9A). Although there were no significant differences among treatments for fungal damage ($p = 0.89$) and combined fungal and insect damage ($p = 0.47$), there were marginal differences in insect damage ($p = 0.07$), as JS sustained marginally lower damage than C ($p = 0.057$). Although not significant, S also showed a trend of lower insect damage levels (Fig. 9A; $p = 0.14$). The M treatment was only assessed for fungal damage, as it was excluded from insect bioassays due to limited numbers of adult apple maggot flies.

Pupal mass and emergence were also evaluated separately, and while there were no differences in pupal mass across treatments ($p = 0.99$, Fig. 10B), there were lower emergence levels in the JS treatment compared to the control ($p = 0.045$, Fig. 10A).

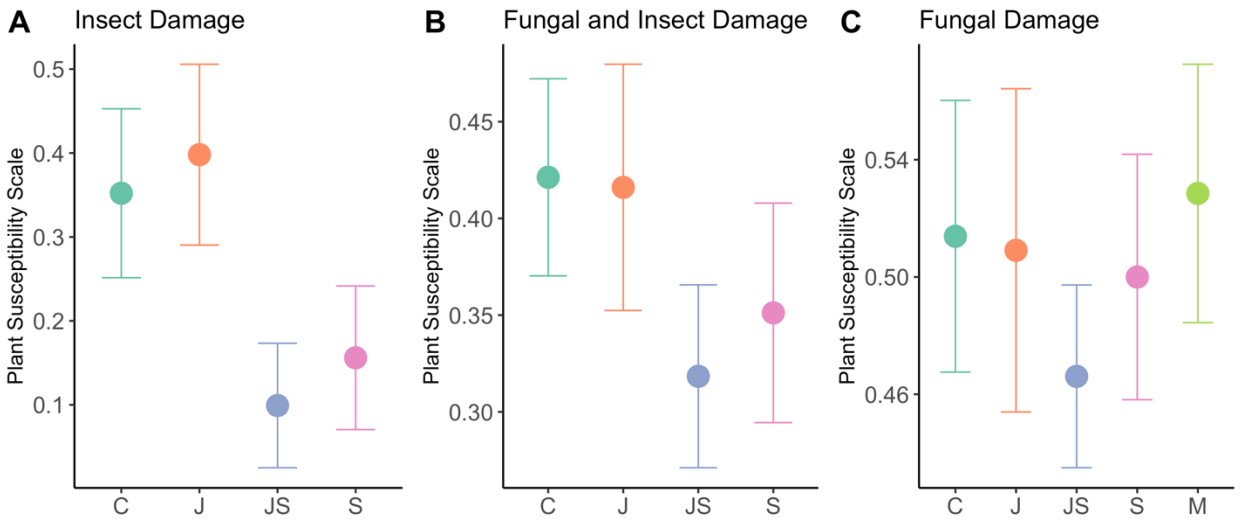

Figure 9. Fruit susceptibility to A) insect damage alone, B) combined fungal and insect damage, and C) fungal damage alone after applications of control (C), jasmonic acid analogue (J), combined jasmonic acid and salicylic acid analogues (JS), salicylic acid analogue (S) and melatonin (M) treatments. The plant susceptibility scale was devised by dividing pupal weight and fungal lesion widths by the maximum values, and taking the mean to assess fungal and insect damage together. Bars indicate standard error.

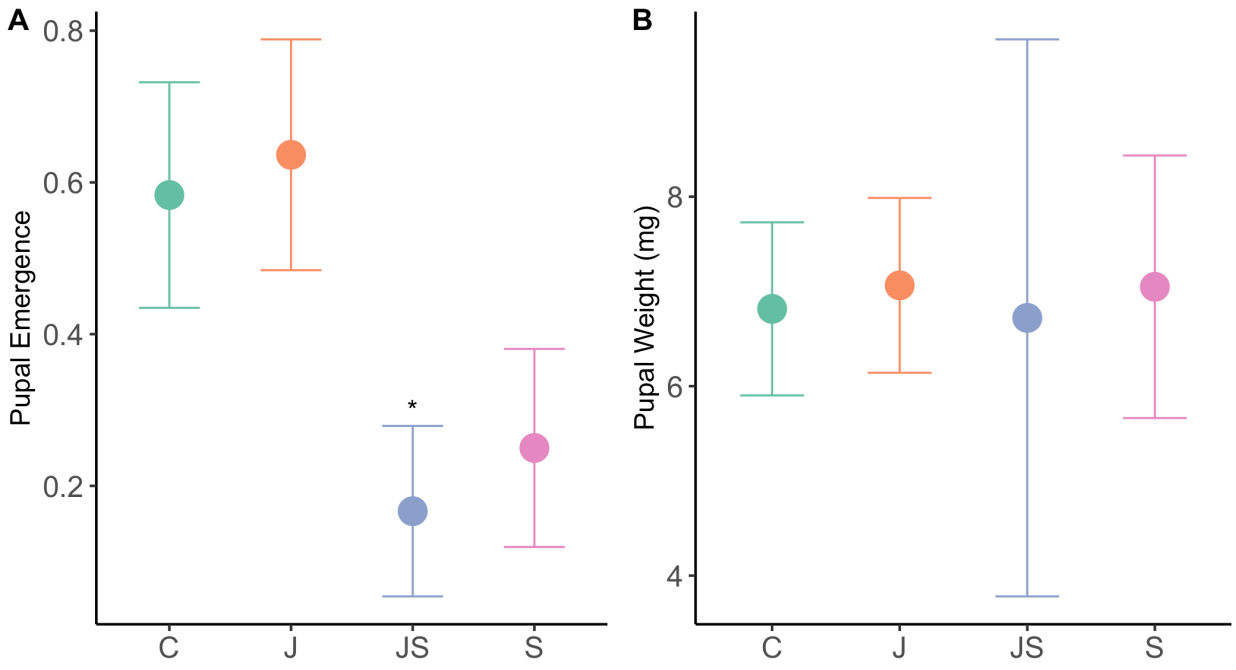

Figure 10. Pupal A) emergence and B) weight (mg) for fruits exposed to insects alone after applications of control (C), jasmonic acid analogue (J), combined jasmonic acid and salicylic acid analogues (JS) and salicylic acid analogue (S) treatments. Bars indicate standard error. Asterisks indicate a significant difference relative to the control treatment.

Discussion

Overall, we found no differences in fruit phenolic chemistry among treatments. This lack of effect may have been driven by a variety of factors, such as timing and dosage of hormone application or the stage of fruit development. More importantly, however, this lack of effect may simply illustrate that the success of exogenous hormone application in inducing plant defenses may be highly variable and dependent upon cultivar type or environmental conditions, which has important implications for utilizing these elicitors as a management strategy. Thus, understanding the primary mechanisms behind this variation should be an area of future focus. However, despite this lack of strong treatment effects, the combined JS hormone treatment, and marginally the S treatment, decreased insect performance by inhibiting larval emergence, potentially caused by limiting oviposition success. Overall, more work needs to be done to improve our understanding of the variability that may limit the efficacy of phytohormone applications in inducing plant defenses, as well as to examine the potential trade-offs that exists

between hormone pathways during defense responses to multi-antagonist attack and implications of these trade-offs for use of elicitors as a more sustainable agricultural strategy.

Defense Elicitor Treatments

Overall, treatments caused no significant changes in apple fruit chemistry in terms of phenolic concentration, diversity, or upregulation of individual compounds (all $p > 0.22$; Figs. 3,5,6). There are a variety of factors that may explain this lack of effect, including the timing of treatment application. Samples were collected five days after treatments were applied, which was anticipated to be close to the point of peak induction based on Actigard® 50WG directions for use, which predicted the highest level of efficacy after 4 days, (Syngenta Inc., 2020), and on prior studies, which typically documented responses after 3-5 days (Schweiger et al. 2014; Ziadi et al. 2001; Thiruvengadam et al. 2015; Cipollini and Sipe 2001). Furthermore, effects were sometimes reported up to 20 days after initial application (Yin et al. 2013), although they have also been observed to drop off after 9-12 days for some activators (Cavalcanti et al. 2006). As the timing of our treatment applications and sample collection fell well within this range, the timing of sample collection seems unlikely to be the driving factor in negating treatment effects; however, the precise window for detecting the chemical response induced by these activators remains unknown, and thus it is possible that the timing of our sample collection fell outside this range.

In this study, we attempted to identify the approximate range of peak induction for each activator by analyzing the phenolic content of hormone-treated fruits compared to control fruits harvested from the same tree after 1,2,3,4,5,8, and 10 days following application. However, although there were no clear temporal effects for most treatments, there was a decline in phenolics over time for skin tissues in the JS ($p = 0.01$), and marginally, S treatments ($p = 0.06$), with a slight increase towards the end of the sampling period for JS-treated skins ($p = 0.01$; Fig. 8). It is possible that any temporal induction trend was masked by the selection of a different apple each day, creating a high level of baseline variation; this seems to be supported by the equally variable concentrations in control fruits (Fig. 8). Indeed, future work exploring intra-tree variation of fruit chemistry would prove valuable to understanding tree defense allocation strategies, and would allow for the development of methods for maximizing treatment benefits. In this study, however, the overall lack of variation in phenolic content between treated and

untreated fruits suggests that there was simply no induction effect strong enough to detect; thus, we were unable to identify patterns of temporal variation among the different activators.

In addition to temporal effects arising from the timing of sample collection, the stage of fruit development during sampling may also have influenced treatment success. An important hormonal change for fruits that occurs early in the season is "June Drop," during which trees drop excessive fruit that would prove too costly for the tree to support. This process involves an interplay among phytohormones such as IAA (Indole-3-Acetic Acid), abscisic acid, and ethylene (Bangerth 2000), the latter of which is also known to interact with the JA pathway (Leon-Reyes et al. 2010a). Later in the season, hormone production becomes important again during the fruit ripening process, which involves multiple hormonal changes and shifts in concentrations of phytochemicals such as volatiles (Osorio and Fernie 2013) to encourage consumption by seed dispersers (Nevo et al. 2018; Valenta et al. 2013). This pattern has been observed by monitoring intra-fruit levels of endogenous JA and anthocyanins, which have been shown to peak twice: once during early cell division and again during fruit ripening (Fan et al. 1998). Our experiment took place during late June and early July in an attempt to avoid the chemical changes that occur in fruit tissues during tree self-thinning and fruit ripening. However, it is possible that the effectiveness of treatment applications may depend on the stage of fruit development and its underlying natural chemical processes. As most work has focused on herbaceous crop species or post-harvest fruit, this complexity may have been thus far overlooked.

It is well-documented that plants are sensitive to the dosage of elicitors that are applied, and that the level of the defensive response is dose-dependent (Karban and English-Loeb 1988; Enyedi et al. 1992). Sometimes, different concentrations can even result in opposing effects; for example, Fariduddin et al. (2003) reported that low doses of SA improved overall plant health in *Brassica sp.*, but that higher concentrations proved injurious. Although the dosages used here have been applied previously with success in apples, it is possible that variable factors such as environmental conditions, plant history, cultivar type, and historic defensive pressures, could cause a change in the concentration levels required to induce a response. Furthermore, for many of the studies in which these elicitors proved effective, treatments were applied with more frequency than a single application (Zhang et al. 2011; Thaler et al. 1996; Schweiger et al. 2014); thus, perhaps the single treatment applied here was not sufficient to induce a defensive response. This may have been particularly true for the J treatment, which is typically applied in multiple

treatments during fruit ripening (Fine Americas Inc. 2014). Thus, hormone success could potentially have been improved by applying more treatment applications and utilizing a detergent to help the hormone pass the fruit skin barrier and activate defenses. In order to assess dosage effects, however, we would have needed to test a wide range of concentrations; however, due to a limited sample size arising from a lack of fruit production, we were unable to test multiple dosage regimes for each of the four elicitor treatments.

Alternatively, it is possible that the use of synthetic analogues instead of pure JA and SA in this experiment could have been a factor in the lack of induction effects. However, this seems unlikely, as S is a commercial fungicide product that contains the active ingredient of acibenzolar-S-methyl, which has been widely reported to defend against a diverse set of fungal pathogens across crop species, including apples (Walters et al. 2013; Dugé de Bernonville et al. 2014). However, the J treatment of Blush2X® was designed to function not in defense, but rather in color enhancement, and therefore it is possible that this activator plays a minimal role in the production of defensive chemical compounds. Furthermore, Blush2X® is known to increase the production of anthocyanins, which are synthesized at the end-point of the flavonoid pathway (Gould et al. 2000). Therefore, an increased production of anthocyanins could alter the synthesis of other phenolic compounds, particularly other flavonoids. However, preliminary evidence suggests that this product can also be used to increase pest and pathogen resistance in apples (Whitehead and Poveda, unpublished). Furthermore, its active ingredient, prohydrojasmon, has been used effectively as a defense elicitor (Azis et al. 2019; Uefune et al. 2014). Thus, it seems more likely that the lack of treatment effects was caused by factors other than the use of these commercial products.

Additionally, it may be that the approach of focusing on one target compound class, phenolics, was not sufficient to detect differences among treatments. In fact, a previous study found that an analysis of targeted metabolites alone revealed only marginal trends, while untargeted metabolic fingerprinting revealed distinct differences among treatment groups (Sutter and Müller 2011). Thus, our treatments may have induced distinct chemical or physiological changes that were not detectable with only an analysis of phenolic compounds. This is further supported by the fact that melatonin, acibenzolar-S-methyl, and prohydrojasmon have all been shown to increase phenolic content (Barilli et al. 2010; Azis et al. 2019; Okatan et al. 2018); if phenolics are common target end products among all three hormone pathways, a comparison of

phenolic content may not reveal any treatment differences. However, this does not explain why the levels of phenolic compounds were not elevated in the treatments compared to the control.

A final explanation may be that the lack of treatment effects was driven by underlying variation in factors influencing plant response, such as environmental conditions, plant genotype, cultivar, crop nutrition, and the level of natural induction already present in plant tissues (Walters et al. 2013). For example, treatments have been to show to vary in effectiveness by plant species or cultivar: the application of exogenous SA defended well against fire blight (*Erwinia amylovora*) in pears but not in apples (Ghahremani and Abdollahi 2011), and SA prevented oviposition of herbivorous mites (*Tetranychus urticae*) in one cultivar of tomato, but not the other (Smart et al. 2013). Furthermore, there have been instances when treatments make no difference because of high levels of underlying induction (Pasquer et al. 2005), suggesting that plant defenses were already induced and could not be activated further. Environmental conditions have also impacted application success; for example, one study showed that JA induction of defensive compounds was effective only under non-drought conditions (Delano-Frier et al. 2004). As an experiment that took place in the field instead of in a controlled laboratory setting, any of these variable factors could have prevented the treatment-induced activation of fruit defenses. In short, although the lack of treatment effects may have been driven by a myriad of aforementioned factors, such as timing, stage of fruit development, and dosage, it may also simply be that application success is variable and dependent upon cultivar type or environmental conditions. This potential variability has important implications for understanding how to best utilize these elicitors as a management strategy, and should be an area of future focus.

Although there were no significant differences among treatments, there did seem to be a marginal trend of higher chemical diversity in skin samples treated with JS (Fig. 3; $p = 0.07$), as predicted. Higher chemical diversity was expected after application of the JS treatment, because although the crosstalk between the J and S pathways may inhibit the production of some compounds, it may also promote a synergism that could increase overall chemical diversity (Schweiger et al. 2014). This trend, if real, suggests that the simultaneous application of J and S could result in synergistic effects that may provide a broader range of defenses that would prove useful to plants facing multi-species attack. However, as this trend was non-significant, more work must be done before accepting this hypothesis.

Additionally, we assessed the possibility that, given the slight visual trend of opposite patterns of total phenolic concentrations in skin and pulp tissues (Fig. 3), treatments could cause an allocation shift of defenses, in which phenolic production is moved from the skin to the pulp region after treatment application in order to protect the fruit interior from rot or tunneling larvae, which may both extend into or feed on the inner pulp tissue. To test this, we compared the ratio of phenolic concentrations found in the pulp relative to the skin for each sample, which, although not a true test of allocation adjustments, could still reveal changes in spatial patterns of phenolic production within different fruit tissues. However, we found no differences among treatments ($p = 0.94$), although we observed again a visual trend of higher pulp to skin ratio in the JS treatment compared to the control (Fig. 4). This trend, although not significant, suggests that defense allocation shifts may be occurring within fruit tissues after treatment applications. Indeed, elicitor treatments have been shown to cause shifts of chemical defenses to specific plant parts (Moreira et al. 2012), although a fine-scale analysis of treatment impacts on fruit tissues has not been conducted. While it should be noted that the trend was not significant and that the use of composite samples from three different fruits may have masked our ability to detect shifts from skin to pulp tissues in an individual fruit, these trends still offer an interesting new direction of inquiry regarding the allocation effects that may arise from these treatments.

Pest and Pathogen Bioassays

Interestingly, although the elicitor treatments caused no significant effects on phenolic chemistry, there were effects on insect performance. Contrary to expectation, J did not reduce insect performance, and sustained damage levels comparable to the control treatment (Fig. 9B). However, the JS treatment caused a marginal reduction in insect performance ($p = 0.058$, Fig. 9B) compared to the control, while S caused a similar trend, although it was not significant ($p = 0.14$, Fig. 9B). The impact of the M treatment on insect performance could not be assessed due to a limited insect sample size. The low values in the rating scale of plant susceptibility to insects for the JS treatment was due its significant effect on decreasing emergence rates ($p = 0.045$), as it caused no effect on pupal weight ($p = 0.96$). This decreased emergence rate was likely due to lower oviposition success, larval survival, or both, but as these variables were not measured, it is unclear which caused the emergence pattern. However, given that pupal weight was unaffected by treatments, decreased oviposition rates may better explain this pattern than larval survival. In

contrast to these effects on insects, fungal growth was not affected by treatments ($p = 0.89$). When fruits were exposed simultaneously to the insect and fungal pathogen, both the JS and S treatments appeared to perform best (Fig. 9B), although again this was not significant ($p = 0.47$) and was driven primarily by their effectiveness against insect damage (Fig. 9B). Thus, the S component of the JS treatment was likely solely responsible for reducing insect performance levels, given the ineffectiveness of the J treatment.

These results contrasted with the expectation that S and M would perform best against the fungal pathogen, while J would provide the best defense against the insect pest. Meanwhile, JS was predicted to defend most effectively against both attackers simultaneously at the cost of effectiveness against individual attack (Fig. 1). However, our findings here suggest that the J and M treatments had little impact on fruit defenses, while the S and JS treatments induced some response that limited insect performance but had negligible effects on fungal growth. Although the JS treatment displayed trends of inducing fruit chemical changes, such as marginally increasing the diversity of phenolic compounds in skin, these trends were slight and non-significant; thus, these impacts on insect performance were likely driven by an effect of the S and JS treatments other than changes in fruit phenolic chemistry. One potential way that treatments could alter fruit susceptibility to insect damage is by altering fruit sugar content. However, our analysis of Soluble Solids Content (SSC) revealed no significant differences among treatments (Fig. 7). Thus, the decreased larval emergence from JS-treated fruits that most likely resulted from reduced oviposition success must have been caused by physical or chemical factors other than phenolics or sugar content. It is possible that the JS, and to a certain extent, the S, treatments may have reduced oviposition rates through the emission of volatile compounds or alterations to fruit surface structure, which were not examined in this study.

All treatments other than JS and S did not affect insect or fungal performance, likely explained primarily by the lack of induced phenolic defenses following elicitor applications. However, it may also be that the separation of the fruit from the tree decreased its defensive ability; indeed, it has been suggested that abscessed fruits are nutrient-starved, and less able to allocate carbon to the production of defensive phytochemicals (Romero et al. 2020). Furthermore, it should also be noted that the pest and pathogen used in this study may not be the ideal for testing JA and SA-specific effects on target antagonists. Bitter rot is a hemibiotrophic pathogen, which means that it necrotizes some plant tissues but not others (Peres et al. 2005).

The SA pathway is suggested to defend best against biotrophic pathogens, while the JA pathway defends best against necrotrophic antagonists (Glazebrook 2005); thus, both hormones may in fact defend effectively against this pathogen. Indeed, exposure to bitter rot has been shown to activate both the SA and JA pathways in strawberries (Amil-Ruiz et al. 2016). Furthermore, the designations between JA and SA-specific targets are not always rigid; in one study, SA and JA were shown to reduce damage from both types of antagonists that were selected to activate the two different pathways (Schweiger et al. 2014). Thus, although selecting the appropriate target antagonist for each hormone pathway should be attempted in these types of studies, it should also be noted that there may be overlapping effects from both pathways. Additionally, the insect pest that was selected, the apple maggot, is an apple specialist. Specialists can typically overcome the induced defenses of their host plant that are effective against generalist insects. In fact, specialists often rely on their host's production of defensive compounds, sometimes accumulating them to produce their own defensive toxins or pheromone signals (Krieger et al. 1971; Miles et al. 2005; Mewis et al. 2002). Thus, it is possible that the defenses induced by elicitor applications may prove effective against a generalist insect, but may be ineffective or even attractive to a specialist like the apple maggot. Thus, the complexities of plant-antagonist interactions need to be fully considered when examining hormone-induced defenses.

Conclusion

Overall, we found no significant differences in fruit phenolic chemistry among treatments, although there were trends of increased chemical diversity in JS skin tissues. This lack of an induced chemical response likely explains the limited impacts of treatments on fruit resistance to fungal and insect damage, although the JS treatment, and to some extent the S treatment, decreased insect performance, specifically insect emergence. This suggests that the JS treatment caused some effect on apple maggot emergence, such as potentially decreasing oviposition success. The lack of overall treatment effects on fruit phenolic chemistry may be explained by a variety of factors, such as the timing of sample collection, the stage of fruit development, or the type of elicitors applied, but may also have simply been driven by variability in plant responses due to underlying environmental conditions, cultivar or crop species effects, or patterns of baseline fruit chemistry. Further investigation is needed to determine which primary factors cause this variability, as it has important implications for incorporating these elicitors into

management strategies. Nevertheless, the non-significant trends of higher skin diversity in the JS treatment, and the fact that JS was the most effective treatment in reducing apple maggot damage, raise tantalizing questions regarding the potential synergism and intra-fruit allocation effects of a combined jasmonic acid-salicylic acid treatment. Improved understanding of how the JA-SA crosstalk may result in a potential tradeoff between targeted defenses against a specific attacker and broader defenses against multiple types of attackers, as well as elucidating the mechanisms behind variation in the success of these hormone applications, may prove invaluable to incorporating elicitors into a more sustainable crop management strategy.

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CHAPTER 3: CONCLUSION

The goal of this project was to provide new insights into the relationship between phytochemistry and biotic interactions in fruit tissues. One consistent finding across both chapters was that fruit phenolic defensive responses, when induced by either damage from biotic interactions or application of defense-activating phytohormones, may be highly variable and difficult to detect due to the complexity and variability of field environments. Variation in induced responses also occurs among cultivars, sampling seasons, fruit tissues, or baseline chemistry of individual fruits, and may be further confounded by the upregulation of these compounds in response to environmental factors, such as UV stress. Thus, identifying the primary factor driving increased phenolic production may be difficult, and more work is needed to elucidate the primary mechanisms behind variability in induced phytochemical responses.

Despite these confounding factors, we observed some effects of biotic interactions on apple fruit chemistry. Notably, pulp tissues, despite containing the lowest baseline levels of phenolic richness and concentrations across tissue types, also constituted the region where the strongest induced defensive responses occurred. In contrast, the phytochemistry and endophyte community of skin tissues appeared to be more strongly affected by environmental factors, such as UV stress, as well as by interactions with the microbial epiphyte community, which contains members that may outcompete other microbial taxa in that region, such as sooty blotch and flyspeck. Finally, seed samples represented a very distinct region of the fruit, containing the highest levels of fungal endophyte diversity and phenolic concentrations. Thus, interaction-driven induced responses in fruits may be tissue-specific, such that phenolic compounds are primarily mobilized to interior pulp tissues, where there may be a need for induced chemical defenses, as this region contains fewer physical barriers or constitutive chemical defenses than skin or seed tissues. This tissue-specific induction is consistent with plant strategies of cost-saving resource allocation; while skin and seed samples may contain higher levels of constitutive defenses in line with their respective roles of providing a barrier against external stressors and surviving dispersal to potentially unfavorable environments, the primary function of pulp tissues is attracting seed dispersers. Therefore, flexibly inducible defenses in pulp tissues would allow a fruit to respond quickly to damage but to reduce phytochemical content when encouraging disperser consumption. Thus, the results from these chapters revealed tissue-specific patterns of fruit chemistry and endophyte composition, indicating that fruit tissues should be assessed

independently when evaluating fruit microbial composition and interaction-driven phytochemical responses.

The relationship between phytochemical and biotic diversity remains an open area of exploration in ecology. We broadly predicted that phytochemical diversity increases with biotic diversity, which was supported by the positive relationship between phenolic diversity and fungal endophyte diversity observed in fruit pulp tissues. However, the mechanism behind this relationship remains unclear; it may be that the presence of certain endophytes induces a defensive response of the host plant, or alternatively, plants may produce phytochemicals that repel or attract certain microbial taxa. Thus, unraveling the mechanism behind this relationship represents an important area of future work. Furthermore, improved understanding of the relationship between phytochemical and biotic diversity may have important consequences for pest management strategies that utilize defense-activating elicitors. In my second chapter, we expected that the activation of multiple defense pathways may produce a higher phytochemical diversity within fruit tissues, offering a broader defense against multiple types of attackers. However, due to the overall lack of treatment effects on phenolic chemistry, this question could not be assessed, and therefore also represents an area of future work.

There are many ways in which the work of these chapters could be expanded in future studies. My first chapter, which examined the effects of distinct treatment-established biotic communities on fruit phenolic chemistry, could be complemented by an experiment applying different types of biotic organisms directly to the fruit, both individually and concurrently, and measuring effects on fruit chemical profiles. These chemical profiles could furthermore be expanded to include analyses of more compound classes and physical traits in addition to phenolic compounds. Additionally, this experiment could also address spatial patterns of induced responses, as it remains unknown whether defenses are localized at the site of damage or are increased throughout the entire fruit. Given our finding that pulp tissues may be an important region for induced defenses, it would also be interesting to examine defense allocation patterns among tissue types following a damage event. Furthermore, additional studies could assess the variability of induced defense patterns across plant taxa or in different environmental settings. Finally, there is a need for studies that examine the chemical interactions between host plants and their associated microbiome, assessing both induced responses to microorganisms within the plant as well as the influence of phytochemicals on microbial taxa, with a focus on how these

interactions may shape both plant traits and microbial community dynamics.

Additionally, the experiment conducted in my second chapter, which examined phytohormone impacts on fruit phenolic chemistry and resistance against multiple types of antagonists, could be repeated to include different hormone dosages and different numbers of application events, as well as a wider variety of cultivars or plant taxa, to improve understanding of the factors that may limit induced responses in fruits. Furthermore, the resistance bioassays could be expanded to include more pest and pathogen species representing both generalists and specialists, and the fruit chemical analyses could evaluate more traits than phenolic compounds and sugar content.

Overall, the relationships between biotic interaction diversity, plant microbiome assembly, and phytochemical diversity represent a complex and open field of exploration, but an important one, as unraveling these interactions may not only yield more sustainable agriculture practices that synergistically improve pest control, enhance crop quality, and provide human health benefits, but may also provide insight into the diverse and complex responses occurring within plant tissues.

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