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ROOT BIOLOGY AND PHYSIOLOGY OF APPLE TREES AS AFFECTED BY FRUIT REMOVAL

A Dissertation in

Horticulture

by

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Abstract. In perennial horticultural crops, the balance between carbohydrate supply and demand is notable because of the high carbohydrate demand required for fruit development. Common management practices that manipulate aboveground growth such as fruit removal, are used to enhance fruit quality, return bloom, and ease of harvest. However, many of the underlying mechanisms behind above- and belowground physiological responses and effects on carbohydrate partitioning remain unknown. Moreover, although the fine root system is critical to water and nutrient acquisition, characterization of absorptive fine roots in mature woody plants is challenging.

For apple (*Malus* x *domestica* Borkh.), crop load management with chemical thinners continues to be a challenge. Early season carbohydrate supply affects fruitlet competition and is assumed to influence efficacy of chemical thinners. The effect of carbohydrate reserves on early season glucose and starch concentrations and the response to chemical thinning were evaluated in mature 'Golden Delicious' apple trees on M.26, M.9, and G.16 rootstocks. In 2013, fruit removal at 29, 125, 154, and 182 days after full bloom was used to alter tree carbohydrate reserves (starch). In 2015, crop density of 2014 was used to alter tree carbohydrate reserves. In 2014, a water control and a chemical thinner of 0.95 L of Carbaryl 4L, 0.95 L Ultrafine oil, and Fruitone N (1-Napthaleneacetic acid) at 15 $mg \cdot L^{-1}$ in 379 L of water were applied to apple trees when fruitlets were 10 to 12 mm in diameter. In 2015, a water control and a chemical thinner of 0.95 L of Carbaryl 4L and Fruitone N (1-Napthaleneacetic acid) at 15 $mg \cdot L^{-1}$ in 379 L of water were applied to apple trees when fruitlets were 10 to 12 mm in diameter. In 2014 and 2015, shoots containing 1- and 2-year-old wood were sampled at different phenological stages from early spring dormancy to 10 days after thinner application to measure total nonstructural carbohydrate

concentrations. Glucose and starch concentrations in December 2013 were not affected by fruit removal date, but total nonstructural carbohydrates declined from early spring dormancy to thinning time in 2014 and 2015. Fruit removal treatments in 2013 and crop density in 2014 had little effect on early season glucose and starch concentrations in 1- and 2-year-old wood in 2014 and 2015. In 2014, fruit set (fruit per 100 flower clusters) on non-thinned trees was positively and linearly related to glucose concentration in 2-year-old wood the day before thinning; however, fruit set on thinned trees was not related to early season glucose concentration. Fruit set was not related to early season starch concentration of 2-year-old wood across all sampling dates regardless of thinning. In 2015, fruit set was not related to glucose and starch concentrations at early season dormancy or thinning time regardless of thinning. Fruit set in 2015 was negatively and linearly related to crop density in 2014 for non-thinned and thinned trees.

In addition to the high carbohydrate demand required for fruit development, apple trees allocate available carbohydrates for belowground processes such as nutrient foraging. Belowground processes such as root production and nutrient foraging require energy to take up nutrients and to support interactions with beneficial microbes such as mycorrhizal fungi. To investigate how carbohydrate availability affected nutrient foraging of ‘Golden Delicious’ trees with or without fruit, fine root and arbuscular mycorrhizal fungal growth was compared in unfertilized soil and localized nitrogen (N)-rich patches (containing inorganic or organic sources of N). Fruit removal enhanced root production compared to fruiting trees across all N treatments. For fruiting trees, roots proliferated more in the inorganic-N patch than in unfertilized soil or the organic-N patch. For non-fruiting trees, root proliferation was similar regardless of N addition. Arbuscular mycorrhizal extramatrical-hyphal biomass was not affected by fruit removal but was greater in

the organic-N patch than in the inorganic-N patch or unfertilized soil. Arbuscular mycorrhizal fungal colonization of apple roots was modestly affected by fruit removal and N treatments, and non-mycorrhizal fungal colonization was unaffected by treatments. Apple trees may manipulate root foraging strategies more than mycorrhizal foraging if carbohydrate availability is limited by fruiting.

Fine roots of woody plants have traditionally been classified using an often arbitrary diameter cutoff approach, such as less than 2 mm. This approach, however, commonly includes both non-woody and woody roots. Alternatively, a functionally based root-order approach can be used to identify absorptive fine roots. The utility of this approach was examined for the horticultural fruit and nut crops: apple (*Malus* x *domestica* Borkh), peach (*Prunus persica*), grape (*Vitus vinifera*), almond (*Prunus dulcis*), and citrus (*Citrus* x *clementina*). In addition, variation in first- and second-order roots (most distal) was characterized for a wide range of woody horticultural species (33 in total), as diameter variation among species could influence the utility of a diameter cutoff approach, and because diameter has been strongly linked to root function. First-order roots of grape and first- and second-order roots of apple and peach were consistently thin, non-woody, mycorrhizal, and had high N:C ratios. In contrast, fourth and fifth order roots of grape and fifth order roots of apple and peach were woody, non-mycorrhizal, had low N:C ratios, and were thicker than lower order roots. Among the 33 horticultural species, diameter of first- and second-order roots varied about 15-fold, ranging from 0.04 to 0.60 mm and 0.05 to 0.89 mm respectively. The weakness of an arbitrary diameter approach is reflected in comparing, for example, first-order roots of date palm (*Phoenix dactylifera* L.) and lemon (*Citrus x limon* L.), which had diameters as large as fourth-order roots of apple and peach. This research shows that

root order characterization has considerably more utility than an arbitrary diameter approach in the identification of roots with different functions in perennial horticultural crops.

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DEDICATION

This dissertation is dedicated to my family who have inspired and encouraged my life-long desire for learning and exploration. I especially thank my husband and my son, my parents and my brother as well as my grandma for their continued love, support, and prayers. "And I am sure of this, that he who began a good work in you will bring it to completion at the day of Jesus Christ." Philippians 1:6.

CHAPTER 1

Introduction

Above- and belowground physiology and carbohydrate partitioning of perennial horticultural crops is complex and dynamic. The balance between carbohydrate supply and demand is notable in perennial horticultural crops because of the high carbohydrate demand required for fruit production. Common management practices manipulate aboveground growth to enhance fruit quality, return bloom, and ease of harvest; however, many of the underlying mechanisms influencing plant responses to current management practices remain unknown. For example, crop load management of apple (*Malus* x *domestica* Borkh.) by chemical thinning has been investigated for decades. Chemical thinning removes a portion of fruitlets which alters tree growth and carbohydrate partitioning to fruit and shoots (Byers, 2003). However, research has focused primarily on the responses of aboveground growth, and effects of fruit removal on belowground physiology and root interactions with soil microbes are rarely considered. Moreover, meaningful characterization of fine absorptive roots and broad comparisons of root trait variation across common horticultural crops is limited by methodology.

The effect of fruit removal on aboveground physiology of apple. Carbohydrate supply is related to factors that affect photosynthesis such as leaf area, light interception, air temperature, water potential, and nutrient availability as well as carbohydrate reserves (Bloom et al., 1985; Palmer, 1992; Byers, 2002; Wünsche et al., 2005; Zhu et al., 2011; Panzacchi et al., 2012). Carbohydrate demand is largely affected by developmental stage of the tree, structural growth of woody and ephemeral plant tissues, and the reproductive cost of fruit (Wünsche et al., 1996; Lakso et al., 1999a).

The dynamic relationship of sink-source interactions has been observed in apple production where natural tree growth may be suppressed as a result of high reproductive costs. For example, Lakso et al., (1999) estimated that the carbohydrate demand of 300 fruit is 60-80% of the total daily carbohydrate supply in a mature, dwarf apple tree trained to a slender spindle system. Other reports support these findings and estimate that 50-80% of available carbohydrates are available for fruit growth in well-managed dwarf apple trees (Forshey and McKee, 1970; Lenz, 1986; and Palmer, 1988). Common growing practices such as deficit irrigation or limited fertilization promote resource limitations that minimize vegetative growth of a tree and maximize carbohydrate availability for fruit at a time when fruit growth is at a minimum.

The manipulation of normal tree growth and the high level of carbohydrate allocated to developing fruit results in limited resources available for growth of above- and belowground tree organs. For example, Palmer (1992) reported that heavy-cropping ‘Braeburn’ trees produced almost twice as much total dry matter per unit leaf area compared to non-fruiting trees, although much of this dry matter was bound in fruit. In non-fruiting trees, 37% of dry matter was partitioned to leaves while only 16% was partitioned to leaves in heavy-cropping trees. Heim et al. (1979) also reported that leaf dry matter was 17% in non-fruiting trees and only 12% in heavy-cropping trees. These differences may be accounted for by variation in rootstock and scion combinations and thinning conditions; however, the high carbohydrate demand of cropping remains evident.

Apple trees have a remarkable ability to meet the high carbohydrate demand for fruit development plus tree growth to sustain high cropping efficiencies over the life of the orchard.

This may be due to regulation of photosynthetic efficiency. For example, whole-canopy photosynthetic rates were higher in cropping trees than non-cropping trees (Jones, 1981; Palmer, 1992). DeJong (1987) reported similar results for peach as well as enhanced uptake of CO_2 during periods of high dry matter accumulation. In addition, Wünsche et al. (2005) reported that net carbon exchange rates (NCER) of heavy-cropping trees was 40-60% higher between 75 days after full bloom (DAFB) and harvest at 173 DAFB, and light capture efficiency and activity of PSII was enhanced compared to non-cropping trees. However, when developing shoots and fruit compete for available carbohydrates, the photosynthetic capacity of the tree canopy is often a limiting factor. Early-season leaf development is rapid, but it may be insufficient to sustain the high carbohydrate demand for fruit and shoot development resulting in carbohydrate shortage and reduced fruit set (Bepete and Lakso, 1998; Zhou et al., 2008; Wünsche et al., 2005).

The balance between carbohydrate supply and demand is particularly important for crop load management of apple because apple trees set far more fruit than desired each year. If the number of apples on a tree is too high, fruit will be small and poorly colored, and return bloom will be low the following year. Therefore, a portion of fruitlets are removed each year to produce high quality fruit of adequate size and to enhance return bloom the following year. To manage crop load, chemical thinning compounds including photosynthetic inhibitors, pesticides, and plant hormones have been experimentally applied to apple trees to evaluate their ability to induce fruit abscission. Most chemical thinners appear to be most effective, when fruit are about 10-12 mm in diameter and carbohydrate demand is high. At this time, carbohydrate reserves are assumed to be low and developing leaves and shoots provide only a limited supply of assimilate. Therefore, the balance between carbohydrate demand from fruitlets and tree growth and the carbohydrate

supply available from actively photosynthetic tissue plus reserve carbohydrates is thought to be at a critical level to support growing fruit. Moreover, environmental factors may affect early season carbohydrate supply and the efficacy of thinners in the context of tree carbohydrate status. For example, high light conditions and low temperatures are associated with high photosynthetic rates, low respiration, and high tree carbohydrate status. This is predicted to suppress the response to chemical thinners resulting in less fruit abscission and higher fruit set. In contrast, low light conditions and high temperatures are associated with low photosynthetic rates, high respiration rates, and low tree carbohydrate status. This is predicted to enhance the thinning response resulting in more fruit abscission and lower fruit set. However, tree responses to chemical thinners and resulting fruit set are highly variable depending on orchard site and year. In addition, observed fruit abscission often differs from expected abscission based on model predictions of tree carbohydrate status using the Cornell Apple Carbohydrate Thinning (CACT) Model. One reason for this variability in response to chemical thinners may be that carbohydrate reserves have a greater impact on early season carbohydrate supply than is currently accounted for by the CACT Model. However, the importance of carbohydrate reserves on the response to chemical thinners has not been critically evaluated.

The effect of fruit removal on belowground physiology of apple. Due to the high energy cost of apple development and shoot growth aboveground, little energy is available for belowground processes and root production (Li et al., 2003). For example, heavy-cropping 'Braeburn' apple trees partitioned only 2% dry matter to roots while non-fruiting trees partitioned 13% dry matter to roots (Heim et al., 1979 and Palmer, 1992). This suggests that almost 70% of dry matter was partitioned to fruit in heavy-cropping trees. Similarly, root biomass and production were

suppressed by cropping in apple (Maggs, 1963; Atkinson et al., 1999; Yao et al., 2009) and citrus (Duncan and Eissenstat, 1993). With such limited energy available belowground, how can roots efficiently forage for nutrients and sustain the high demand for water and nutrients needed for apple development over the life of an orchard?

Root foraging for nutrients is an energy intensive process, and plants need to balance the energy required for root production with the return of water and nutrient acquisition (Bloom, 1985). In response to nutrient addition, root production, root lifespan, and uptake kinetics are enhanced (Drew, 1975; Bassirirad et al., 1993; Linkhor et al., 2002). However, root responses may differ due to nutrient source (e.g. mineral or organic) in localized nutrient-rich patches (Liu et al., 2015; Cheng et al., 2016). In apple production systems, the uptake of N, which is often a limiting resource, is important to maintain tree health and sustain fruit production over the life of the orchard (Peter, 2018); thus, understanding strategies trees use to enhance nutrient foraging is important particularly when carbohydrate availability is limited by cropping.

In addition to altering root responses, plants may also interact with beneficial microbes such as arbuscular mycorrhizal (AM) fungi (Smith and Read, 2008) to enhance nutrient foraging. Arbuscular mycorrhizal extramatrical hyphae proliferate in the soil to forage for nutrients, and hyphae transfer nutrients to the host plant in exchange for carbohydrates. Arbuscular mycorrhizal fungi may also aid plant roots by protecting them from pathogens through competition, induction of plant-defense pathways, enhanced nutrient levels needed for plant defense compounds, or the production of antimicrobial compounds that suppress pathogen infection (Newsham et al., 1994; Newsham et al., 1995; Smith and Read, 2008). In apple, Resendes et al. (2008) reported that

young roots colonized by AM fungi had higher root growth rates than uncolonized roots or roots colonized by non-mycorrhizal fungi. They hypothesized that AM fungal colonization may be linked to carbohydrate resources of individual roots; however, if carbohydrate availability to roots and AM fungi is suppressed by fruit development in apple, symbiosis with AM fungi may be limited.

Root trait variation of perennial horticultural crops. Root responses to abiotic and biotic factors have been investigated in perennial horticultural crops with emphasis on site, water and nutrient availability, cultivar, and rootstock (Eissenstat, 1991; Green and Clothier, 1999; Rosecrance et al., 1996). However, our understanding of environmental and cultivar effects on root physiology remains limited, particularly for the most distal, absorptive, fine roots. Absorptive fine roots exhibit traits associated with water and nutrient absorption such as thinner diameter, absence of secondary thickening, mycorrhizal colonization, high N:C ratios, and short lifespans while woody roots associated with water and nutrient transport are characterized by thicker diameter, secondary thickening, absence of mycorrhizal colonization, low N:C ratios, and long lifespans. Rapid identification of roots with absorptive or transport functions has been challenging. Traditionally, researchers classified fine roots using an often arbitrary root-diameter cutoff where all roots less than 2 mm were characterized as fine roots. However, root traits such as root diameter, can vary considerably across species. Thus, characterization of fine root function using a 2 mm diameter cutoff approach, for example, may result in erroneous estimates of absorptive fine roots and misrepresent root system responses across horticultural species.

Therefore, the objectives of this work were to 1) evaluate the assumptions of the CACT Model for fruit thinning by a) evaluating the relationship between reserve carbohydrate levels in 1- and 2-year-old shoots and previous season crop load, b) determine the relationship between reserve carbohydrates in shoots and fruit set, and c) determine if the relationship between reserve carbohydrate concentrations in shoots is altered by application of a fruit thinning treatment; 2) examined how apple fruit removal affects root and mycorrhizal fungal proliferation in nutrient "hot spots" in the soil; 3) investigate the utility of using a functional root-order approach compared to an arbitrary diameter cutoff to characterize fine roots of different functions in the horticultural crops: apple, peach, grape, almond, and citrus; and 4) to characterize the variation in root diameter of 1st- and 2nd-order roots of a wide range of perennial horticultural crops.

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CHAPTER 2

The Effect of Fruit Removal on Stored Carbohydrates and the Response to Chemical Thinning in Apple

Abstract. Apple (*Malus* x *domestica* Borkh.) crop load management with chemical thinners continues to be a challenge for apple production systems. Early season carbohydrate supply affects fruitlet competition and is assumed to influence efficacy of chemical thinners. The effect of carbohydrate reserves on early season glucose and starch concentrations and the response to chemical thinning were evaluated in mature 'Golden Delicious' apple trees on M.26, M.9, and G.16 rootstocks. In 2013, fruit removal at 29, 125, 154, and 182 days after full bloom was used to alter tree carbohydrate reserves (starch) in individual trees. In 2014, a water control and a chemical thinner of 0.95 L of Carbaryl 4L in 379 L of water, 0.95 L Ultrafine oil in 379 L, and Fruitone N (1-Napthaleneacetic acid) at 15 $mg \cdot L^{-1}$ was applied to trees when fruitlets were 10 to 12 mm in diameter. In 2014, shoots containing 1- and 2-year-old wood were sampled at different phenological stages from early spring dormancy to 10 days after thinner application to determine glucose and starch concentrations. Crop density in 2014 was used to alter early-season tree carbohydrate reserves in 2015. Thinning treatments included a water control and a chemical thinner of 0.95 L of Carbaryl 4L in 379 L of water and Fruitone N (1-Napthaleneacetic acid) at 15 $mg \cdot L^{-1}$ was were applied in 2015 as described previously, and shoots were sampled at early spring dormancy and thinning time to determine glucose and starch concentrations in 1- and 2-year-old wood. Glucose and starch concentrations declined from early spring dormancy to thinning time in 2014 and 2015. Fruit removal treatments in 2013 and crop density in 2014 had

little effect on early season glucose and starch concentrations in 1- and 2-year-old wood in 2014 and 2015. In 2014, fruit set (fruit per 100 flower clusters) for non-thinned trees was positively related to glucose concentration in 2-year-old wood sampled the day before thinning; however, for thinned trees, fruit set was not related to glucose concentration at any sampling dates. Fruit set was not related to early season starch concentration for 2-year-old wood across all sampling dates regardless of thinning. In 2015, fruit set was not related to glucose and starch concentrations at early season dormancy or thinning time for non-thinned and thinned trees. However, fruit set in 2015 was negatively and linearly related to crop density in 2014 for non-thinned and thinned trees. This work suggests that the tree responses to chemical thinners may not be directly related to glucose and starch concentrations in 1- and 2-year-old wood of apple.

Additional index words. chemical thinning, carbohydrate reserves, crop density, starch

Introduction

Apple (*Malus* x *domestica* Borkh.) crop loads are managed to balance fruit yield and fruit size, produce high quality fruit, and enhance return bloom (Ferree and Warrington, 2003). One way to manage apple crop load involves fruit removal using chemical thinners; however, chemical thinning can be a challenging and sometimes unpredictable practice (Byers et al., 1990a; Byers et al., 1990b; Byers, 2003; Greene et al., 2011). Application of chemical thinners can effectively reduce fruit set (the number of fruit per 100 blossom clusters). Unfortunately, thinning can be unpredictable, leading to excessive fruit abscission and reduced yield or inadequate fruit

abscission and excessive yield often with poorer quality fruit. Thus, there is a need to more mechanistically understand tree physiology in relation to chemical thinning.

The response of apple trees to chemical thinner application is currently thought to be related to the carbohydrate supply and demand of the tree. Carbohydrate supply is highly dependent on factors that affect photosynthesis such as leaf area, light interception, air temperature, water potential, and nutrient availability (Byers, 2002; Palmer, 1992; Panzacchi et al., 2012; Wünsche et al., 2005; Zhu et al., 2011). Carbohydrate demand is influenced by the developmental stage of the tree, tree vigor, and fruit production (Wünsche et al. (1996). Apple trees may also shift carbohydrate supply and demand by altering the metabolism of stored carbohydrates or allocation patterns in the tree (Bloom et al., 1985; Corelli Grappadelli et al., 1994). For example, if the carbohydrate status of a tree is low, shoot growth may out-compete fruit growth resulting in reduced fruit set (Bepete and Lakso, 1998). Byers (2003) and Zhou et al. (2008) also reported that when the carbohydrate supply was lower than the demand, fruitlets were highly susceptible to a carbohydrate shortage, and they abscised from the tree. When shoot growth and developing fruit compete for available carbohydrates, the photosynthetic capacity of the tree canopy is often the limiting factor. Lakso et al. (1999) observed that 20% of the leaf area was developed by four weeks after bud break which suggests that leaf development was rapid initially; yet, the leaf area at that time may be insufficient to support the high carbohydrate demand needed for fruitlet development.

The Cornell Apple Carbohydrate Thinning Model (CACT Model; Lakso et al., 1999; Lordan et al., 2019) was developed to predict early season carbohydrate supply and demand of apple trees and the potential response of fruit to the application of a chemical thinner. Using daily light and

temperature measurements, the CACT Model considers leaf area development, daily canopy photosynthesis, respiration rate, and dry matter partitioning to predict periods of surplus, deficit, and the corresponding balance of carbon available for fruit development over the season. In other words, the CACT Model estimates a threshold of available carbohydrate needed for desired fruit set and predicts whether tree carbohydrate status is above or below that threshold. The CACT Model is being used by commercial growers to modify crop load management strategies. However, tree responses to thinners often differ from predicted responses, and thinning the proper amount of fruit continues to be a challenge.

One source of variation in the CACT Model predictions may be due to carbohydrate reserves. Some key assumptions of the model predictions are that fruit are most susceptible to thinners when 1) carbohydrate reserves are highly depleted and 2) carbohydrate availability is low. However, the levels of carbohydrate reserves needed for annual tree growth and fruit set as well as the amount of carbohydrate the tree will store in autumn for future growth is not well understood.

Therefore, the objective of this study was to evaluate a key assumption of the CACT Model by determining the influence of thinning treatments on the relationship between fruit set and carbohydrate levels in 1- and 2-year-old wood. We hypothesize that 1) autumn carbohydrate reserves in the tree could be manipulated by defruiting trees at different times of the season, 2) trees with more stored carbohydrates (starch) from the previous year would also have more carbohydrates available at thinning time than trees with less stored carbohydrates from the

previous year, and 3) trees with more carbohydrates available at thinning time would have higher fruit set after chemical thinning than trees with less carbohydrates available.

Materials and Methods

Field site and experimental design. The experiment used 28 'Golden Delicious' apple trees on three dwarfing rootstocks: M.9NAKBT337 (M.9; moderate vigor), M.26 (high vigor), and G.16 (low vigor). Trees were grown at the Pennsylvania State University Russel E. Larson Agricultural Research Center at Rock Springs, Pennsylvania, USA (40.8˚N and 77.9˚W, elev. 350 m). Trees were planted in 2003, as part of the NC-140 Apple Physiology trial, at 3.5 x 5.5 m spacing. Trees were oriented in a north-south direction and were trained to a vertical axe system. Dormant pruning, fruit thinning, pest control and harvesting were accomplished according to standard cultural practices used in commercial orchards in the region (Peter, 2018). Soil at this site had a pH of 7.1 and was in the Hagerstown series (mesic Typic Hapludalf). Soil organic matter content was 1.7% in the top 0.25 m of the soil profile.

This experiment was a completely randomized design, and we assumed that trees on all rootstocks would respond similarly to treatments. Four fruit removal treatments were designed to manipulate the level of stored carbohydrates in trees available for growth and fruit set the following season. Fruit removal treatments in 2013 were intended to create a range of carbohydrate reserves in the tree to investigate early season nonstructural carbohydrate status and fruit set (fruit per 100 flower clusters) in 2014. In year one (2013), trees were defruited on one of four dates during the growing season to alter the amount of time available for carbohydrate

(starch) accumulation between fruit removal and leaf senescence in the fall. In 2013, seven trees were assigned to one of the four following treatments: 1) *no fruit* (trees defruited on 5 June at 29 days after full bloom (DAFB) when average fruit size was 14.7 mm; 2) *early fruit removal* (fruit were harvested prematurely on 7 – 9 Sept., 125 DAFB); 3) *normal fruit removal* (fruit were harvested on 5 – 7 Oct. when fruit were mature, 154 DAFB); and 4) *late fruit removal* (fruit were removed on 28 – 30 Oct., 182 DAFB). We hypothesized that carbohydrate levels would be highest in trees without fruit followed by trees with fruit removed at early, normal, and late harvest dates, respectively. Although rootstock was assumed to have a negligible effect on treatment responses, randomization of treatments was restricted by assigning at least two trees on each rootstock to each fruit removal treatment. Before harvest, trees with fruit removal treatments of 125, 154, and 182 DAFB had similar crop densities (fruit per cm^2 trunk cross-sectional area; TCSA) of 6.19, 4.22, and 5.7 respectively ($P<0.01$). For early, normal, and late fruit removal treatments, all fruit were harvested and counted. Early season nonstructural carbohydrates and fruit set were also measured in 2015. However, due to differences in return bloom and yield in response to initial fruit removal treatments, crop density in 2014 was used as a predictor for carbohydrate status and fruit set in 2015.

Chemical thinner application in 2014. On 3 June, when fruit dimeter averaged 10-12 mm, a chemical thinner was applied to three of the seven trees of each fruit removal treatment using a hand gun. Randomization of thinning treatment was restricted to include one tree of each rootstock. The thinner included a mixture of 0.95 L of Carbaryl 4L, 0.95 L Ultrafine oil, and Fruitone N (1-Naphthaleneacetic Acid; AMVAC Chemical Corporation, Los Angeles, CA) at 15 $mg \cdot L^{-1}$ in 378.5 L of water, and trees were sprayed to runoff. The remaining trees in each fruit

removal treatment were sprayed with water to runoff using a hand gun. On 10 July, final fruit set was recorded for three randomly selected branches per tree. Additional hand thinning was required on trees that were not sprayed with chemical thinner. It is important to note that the carbon balance model predicted a tree surplus in available carbohydrates and recommended increasing the chemical thinner rate by 30%. The thinner applied in this study was not adjusted according to the recommendations.

Chemical thinner application in 2015. On 29 May 2015, when fruit were 10-12 mm in diameter, thinner application was randomly applied as described above to half of the trees (14) across a range of 2014 crop density values. Water was applied to non-thinned trees as described previously. Randomization of thinning treatments was restricted to include at least 3 trees from each rootstock. As in 2014, the carbon balance model predicted a surplus in available carbohydrates and recommended increasing the thinner rate by 15%. The thinner application in 2015 was not adjusted according to the recommendations.

Shoot sampling. On 9 Dec. 2013 when trees were dormant, three shoots per tree were removed, and shoots contained 1- and 2-year-old wood. One and 2-year old wood was selected for sampling because such shoots were indicators of carbohydrate status in woody tissue (McQueen et al., 2004; Naschitz et al., 2010). In addition, 2-year-old wood supported flowering spurs and was selected as an indicator of wood associated with a higher local carbohydrate demand from fruit than 1-year-old wood. Shoots were transferred to a cooler and briefly stored at 4˚C until collection of subsamples. Subsamples were taken from midsections of 1- and 2-year-old wood

from each shoot and were flash frozen with dry ice, lyophilized at -5˚C for 7 days, and stored at 22˚C until total nonstructural carbohydrate (TNC) extraction.

In 2014, shoots were sampled at different phenological stages or periodically beginning when trees were still dormant until three weeks after chemical thinning. Shoots were sampled on 1 Apr. (early spring dormancy), 19 Apr. (green tip), 2 May (tight cluster), 8 May (pink), 15 May (bloom), 2 June (day before thinning application), and June 10 (8 days after thinner application). At each sampling time, three shoots were sampled per tree, and shoots contained 1- and 2-year-old wood. Subsamples were collected from shoot midsections for each wood age, and samples were processed as described previously. In 2015, shoots were sampled and processed as described above; however, only data for 8 Apr. at early spring dormancy and 29 May at thinning time are reported.

Total nonstructural carbohydrate determination. Freeze-dried shoot samples of 1- and 2-year-old wood were ground using a Wiley model 1 mill with a 2 mm mesh screen. Total nonstructural carbohydrate (TNC) concentrations were extracted from 2013, 2014, and 2015 shoot samples using a modified Somogyi-Nelson reducing sugar determination method (Nelson, 1944; Somogyi, 1952). This method uses enzymatic digestion to rapidly quantify glucose and starch concentration and is a cost-effective approach to analyze many samples. Briefly, two, 5 mg subsamples of 1- and 2-year-old wood from each shoot sample were weighed into 2 mL microcentrifuge tubes for soluble sugar extraction (glucose) and starch digestion. One mL of deionized water was added slowly to each tube to wet the shoot material and tubes were boiled in a hot water bath for 20 minutes. Tubes were removed from the hot water bath and immediately

placed in an ice bath until cool. For the soluble sugar extraction, 100 µL of 0.5 M sodium acetate was added to each subsample. For starch digestion, 100 µL of 0.5 M sodium acetate containing 5 units of amyloglucosidase (E.C.3.2.1.3.) and 2.5 units of α-amylase (E.C.3.2.1.1.) was added to each subsample. Tubes were incubated for 24 hours at 30˚C. Digestion was stopped by boiling samples for five minutes. Tubes were immediately placed in an ice bath until cool.

For colorimetric analysis, 500 µL of Nelson's reagent A was added to soluble sugar and starch extracts. Extracts were diluted as necessary to ensure that sugar samples were within the range of the standard curve (0-120 µg glucose·mL^{-1}). Tubes were boiled for 10 minutes and immediately placed in an ice bath to cool. Once cool, 500 µL of Nelson's reagent B was added to each tube followed by 3.5 mL of water. Samples were vortexed and placed in the dark for 30 minutes. Sample absorbance was read at 520 nm using a UV-1600PC Spectrophotometer (VWR, Radnor, PA). The difference in absorbance between the soluble sugar and starch determination tubes was used to calculate starch concentration for 1- and 2-year-old wood of each shoot sample.

Additional measurements. Trees were pruned in mid-Apr., in 2014 and 2015. To estimate the effect of fruit removal and yield on tree growth, pruning fresh weights were recorded for each tree. In 2014 and 2015, blossom density (blossom clusters per cm^2 branch cross-sectional area) and initial fruit set (fruit per 100 blossom clusters) were counted on three randomly selected branches per tree. In addition, fruit diameter and shoot extension were measured bi-weekly on five randomly selected fruit and shoots per tree. All trees were harvested from 14 - 16 Oct. in 2014 and 5 - 9 Oct. in 2015, and total fruit number and average fruit weight were calculated per

tree. Trunk cross sectional area (TCSA) was also measured yearly at 30 cm above the ground after leaf fall in 2014 and 2015.

Statistical analysis. The treatment structure was originally a 4 x 2 factorial with 4 fruit removal dates (from 2013) and 2 thinning treatments (in 2014) with 3 replications in a completely randomized design. However, at least one tree of each rootstock was assigned to each treatment combination. Due to the limited number of degrees of freedom needed for analysis of variance (ANOVA) for 2014 data, rootstock and interactions involving rootstock were pooled into the error term. In 2014, glucose and starch concentrations, fruit set, crop density, shoot length, and fruit diameter were analyzed by ANOVA using SAS's Mixed Procedure (SAS Institute Inc., Cary, NC). Independent variables included fruit removal treatments, thinning treatments, wood age, and interactions. TCSA in 2014 was analyzed by analysis of covariance (ANCOVA) with 2013 TCSA as a covariate and fruit removal and thinning treatments as indicator (class) variables using SAS's Mixed Procedure.

In 2015, return bloom and fruit set were variable due to effects of biennial bearing; therefore, crop density from 2014 was used as a regressor variable. Thinning treatment, rootstock, and wood age of shoots were used as independent (class) variables to test for glucose and starch concentrations, fruit set, TCSA, crop density, shoot length, fruit diameter, and dormant pruning weights using SAS's Mixed procedure.

Results

Treatment effects in 2013. Trunk cross-sectional area was higher (P<0.01) for trees on M.26 (116 cm^2) and M.9 (96.1 cm^2) than G.16 (67.3 cm^2). Mean glucose and starch concentrations were similar at early winter dormancy in 2013 for trees assigned to all fruit removal treatments and for both wood ages (data not shown).

Nonstructural carbohydrates in 2014. Glucose concentration in 1- and 2-year-old wood increased over time from dormancy to the day before thinning time in 2014 (P<0.01; Fig. 2.1A, B). Glucose concentration in 1- and 2-year old wood was similar at early spring dormancy, tight cluster, and the day before thinning time (P>0.05; Table 2.1). One-year-old wood had a higher glucose concentration than 2-year-old wood at bloom and at eight days after thinner application (P<0.01). Two-year-old wood had a higher glucose concentration than 1-year-old wood at green tip and the blossom stage of pink (P<0.01). At almost all sampling times, glucose concentration was not affected by fruit removal the previous season and wood age (P>0.05). Fruit removal affected glucose concentration only at the blossom stage of pink for both 1- and 2-year-old wood where trees with early and normal fruit removal had higher glucose concentrations than trees with late fruit removal, and no fruit trees were intermediate (P=0.02). The application of a chemical thinner enhanced glucose concentration in 1- and 2-year-old wood sampled at eight days after application (P<0.02).

In contrast to glucose, starch concentration declined in 2014 from early spring dormancy to thinning time in 1- and 2-year-old wood (P<0.01; Fig. 2.1C, D). Starch concentration was similar

in 1- and 2-year-old wood at the blossom stage of pink, at the day before thinner application, and eight days after thinner application. Starch concentration was higher in 2-year-old than 1-year old wood at dormancy, green tip, tight cluster, and bloom. Fruit removal had no effect on starch concentration of 1- and 2-year-old wood at green tip, tight cluster, bloom, the day before thinning, and eight days after thinning in 2014. However, at early spring dormancy, starch concentration in 1- and 2-year-old shoots was higher in no-fruit trees than trees with late fruit removal while trees with early and normal fruit removal were intermediate (P=0.05). In addition, at the blossom stage of pink, trees with early fruit removal had higher starch concentration than no-fruit and late fruit removal trees while trees with normal fruit removal were intermediate regardless of wood age (P<0.01). Chemical thinner altered starch concentration at eight days after application; however, this depended on wood age and fruit removal treatment (P<0.03). For non-thinned trees, starch concentration of 1-year-old wood was lower in no-fruit trees than trees with late fruit removal, and trees with early and normal fruit removal were intermediate. In contrast, starch concentration of 2-year-old wood was lower in trees with early and late fruit removal than trees with no-fruit and normal fruit removal.

Crop density and fruit set in 2014. Fruit removal treatment in 2013 had no effect on crop density in 2014 (P=0.39). Crop density was higher for non-thinned trees than thinned trees (Table 1; P<0.01). Fruit removal treatment had no effect on fruit set (fruit per 100 flower clusters; P=0.12). Non-thinned trees set 73.2 fruit per 100 flower clusters while thinned trees set 8.49 fruit per 100 flower clusters (P<0.01). Since fruit removal treatment had no effect on 2014 fruit set, nonstructural carbohydrate concentrations of 1- and 2-year-old wood at early spring dormancy, bloom, and thinning time were analyzed as potential predictors of fruit set. Fruit set was not

related to glucose concentration in 1- and 2-year-old wood at dormancy or bloom time (P>0.19; Fig. 2.2A). At thinning time, fruit set was positively linearly related to glucose concentration in 1- and 2-year-old wood in non-thinned trees but not thinned trees (P=0.04; Fig. 2.2C). For thinned trees, fruit set was not related to glucose concentration (P>0.31).

In contrast to glucose, fruit set was negatively and linearly related to starch concentration in 1- and 2-year-old wood at dormancy for non-thinned trees (P=0.03; Fig. 2.2B). For thinned trees, fruit set was not related to starch concentration at dormancy regardless of wood age (P=0.54). Fruit set was not related to starch concentration of 1- or 2-year-old wood at bloom or thinning time, regardless of thinner application (P>0.34; Fig. 2.2D).

Tree growth measurements in 2014. Trunk cross-sectional area (TCSA) was not affected by 2013 fruit removal or thinning treatments (Table 1; P<0.01), but in 2014, it was positively and linearly related to TCSA measured in 2013. Shoots were longer on trees with no fruit than late fruit removal trees, and shoot length on trees with early and normal fruit removal was intermediate (Table 1; P<0.01). Shoot length was similar on non-thinned and thinned trees across all fruit removal treatments (P>0.49). Fruit diameter at harvest was larger for thinned trees with no fruit in 2013 than non-thinned trees (Table 1; fruit removal x thinning interaction: P<0.03). Fruit diameter was similar regardless of thinning treatment for trees with early, normal, and late fruit removal.

Nonstructural carbohydrates in 2015. At early spring dormancy, glucose concentration was higher in 2-year-old than 1-year-old wood (P=0.01) but was unaffected by 2014 crop density or rootstock (P>0.38). At thinning time, glucose concentration was lower in 2-year-old than 1-year-

old wood (P<0.01), and trees on M.26 had lower glucose concentrations (12.4 mg g^{-1} dry mass) than trees on M.9 (15.6 mg g^{-1} dry mass) and G. 16 (16.5 mg g^{-1} dry mass; P=0.03). Glucose concentration, at thinning time, was not related to 2014 crop density (P=0.60). In contrast to glucose, starch concentration was similar in 1- and 2-year-old wood for all rootstocks at both early spring dormancy and thinning time (P>0.42). Starch concentration at early spring dormancy or thinning time was not related to 2014 crop density (P>0.13).

Crop density and fruit set in 2015. Crop density was higher for non-thinned trees than thinned trees (Table 2; P<0.01). Crop density in 2015 was negatively and linearly related to crop density in 2014 (P<0.01). Fruit set was not related to glucose or starch concentration of 1- and 2-year-old wood at dormancy or thinning time in 2015 (P>0.11). In contrast to nonstructural carbohydrate concentration, fruit set was negatively and linearly related to 2014 crop density for both non-thinned and thinned trees (Fig. 2.3). In addition, when crop density in 2014 was low, 2015 fruit set was higher in non-thinned than thinned trees; however, when crop density in 2014 was high, 2015 fruit set was similar in non-thinned and thinning (thinning x crop density interaction; (P<0.01).

Tree growth measurements in 2015. Trunk cross-sectional area in 2015 was not affected by thinning and was not related to crop density in 2014 (P>0.33). Trees on M.26 and M.9 rootstocks had higher TCSA than trees on G.16 (P<0.01). Shoots were longer on non-thinned trees on M.26 rootstock followed by M.9, and G.16; however, for thinned trees, shoots were longer on M.9 than M.26 and G.16 (Table 2; thinning x rootstock interaction; P<0.01). Fruit diameter was larger for thinned trees than non-thinned trees across rootstock (P<0.01). Total dormant pruning

weights were higher for trees on M.26 and M.9 rootstocks than trees on G.16 (Table 2; $P<0.01$). Non-thinned trees on M.26 had higher pruning weights than thinned trees, and pruning weights of trees on M.9 and G.16 were similar regardless of thinner application ($P=0.04$).

Discussion

In this study, fruit removal treatments in 2013 and crop density in 2014 were designed to alter carbohydrate accumulation at the end of the growing season and provide a range of carbohydrate reserves available for spring growth and fruit set of apple trees the following season. Previous studies have shown that dry matter accumulation is suppressed by fruiting (Palmer, 1992). Similarly, summer pruning in apple has been used to investigate the effect of reducing the photosynthetic capacity of the tree on carbohydrate reserve accumulation and tree growth the following season (Marini and Barden, 1982). By suppressing the trees' ability to accumulate photosynthate by delayed fruit harvest, higher crop density or removal of photosynthetically active shoots by summer pruning, one would expect a marked reduction in carbohydrate reserves available for the following season. However, woody tissues may have a large capacity to store and recycle carbohydrates to maintain a certain threshold of available carbohydrates making the manipulation of reserves difficult (Greer et al., 2002; Hennerty and Forshey 1971; McQueen et al., 2004). For example, Marini and Barden (1987) reported that bark tissue from dormant pruned trees had more nonstructural carbohydrates in Nov. than summer pruned trees. However, by bloom time the following season, summer pruned trees had 9% more nonstructural carbohydrates than dormant pruned trees.

In our study, fruit removal in 2013 did not influence glucose or starch concentrations in 1- and 2-year-old wood at winter dormancy in 2013 or from early spring dormancy to eight days after thinning in 2014. In addition, crop density in 2014 did not affect glucose or starch concentrations in 1- and 2-year-old wood at early spring dormancy or at thinning time in 2015. Perhaps fruit removal and crop density treatments were not extreme enough to affect late-season carbohydrate accumulation for the following spring. Carbohydrate reserves may also have been stored in other tissues such as the trunk, roots, or older wood compared to 1- and 2-year-old wood. Late-season photosynthate has been shown to accumulate in trunks and roots of apple (Priestly, 1962; Kozlowski, 1962), and carbohydrates are remobilized over the winter and spring and transported throughout the tree to support growth (Sperling et al., 2017). However, differences in carbohydrate status of the tree at bloom time, were expected in the glucose and starch concentrations of 1- and particularly 2-year-old wood due to their close proximity to actively growing shoots and developing fruitlets compared to the trunk or roots.

Alternatively, if trees have accumulated sufficient carbohydrate reserves and excess carbohydrates are available, compensatory growth the following season or enhanced respiration rates (Sperling et al., 2017) that utilize excess carbohydrates might also account for similar glucose and starch concentrations in 1- and 2-year-old wood. For example, deblossoming and early fruit removal of apple trees has been correlated with enhanced shoot length, spur-leaf area, trunk size and root growth (Denne, 1963; Maggs, 1963). In contrast, if carbohydrate reserves are insufficient to support growth, apple trees may adjust photosynthetic rates in response to the sink strength of developing fruit (Wibbe and Blanke, 1995, Palmer et al., 1997; Zhu et al., 2011). For example, fruiting ‘Braeburn’ apple trees on M.26 rootstock, had about 40% higher

photosynthetic rates than non-cropping trees (Wünsche et al., 2005). Therefore, net carbon assimilation could be upregulated or downregulated to meet the tree carbohydrate demand.

We found that date of fruit removal in 2013 had little effect on vegetative and reproductive growth in 2014. Late fruit removal suppressed shoot growth in 2014 compared to the no fruit treatment but had no effect on TCSA or fruit diameter. Also, crop density in 2014 had little effect on dormant pruning weights, shoot length, or fruit diameter in 2015, but was negatively and linearly related to TCSA. In 2015, the higher vigor rootstocks, M.26 and M.9, had longer shoots and greater TCSA than the lower vigor rootstock, G.16 which may have implications for carbohydrate pool size (Caruso et al., 1997; Walcroft et al., 2004).

Starch concentration in 1- and 2-year-old wood declined from early spring dormancy to thinning time in 2014 and 2015; however, fruit set was similar over a range of starch concentrations in 1- and 2-year old shoots at dormancy and thinning time in 2014 and 2015. This supports other findings that carbohydrate reserves are metabolized early in the growing season (Quinlan, 1969; Hansen and Grauslund, 1973), but they may have a minimal role in the supply of carbohydrates after bloom with the development of spur leaves and extension shoots (Hennerty and Forshey, 1971; Loescher and McCamant, 1990; Corelli Grappadelli et al., 1994). This also supports the apple carbohydrate thinning model assumption that stored carbohydrates play a minimal role in the response to chemical thinners (Lakso et al., 1999). Alternatively, other pools of starch storage such as roots or older wood and pool size may contribute more to the available carbohydrate concentration than 1- and 2-year old shoots in early season development. Unfortunately, studies to identify carbohydrate pools and seasonal dynamics in carbohydrate allocation patterns are few

in mature trees (Palmer et al., 1997) and are challenging due to the destructive nature of observations and constraints on labor and resources required for tissue dissection and carbohydrate analysis.

Fruit set was positively and linearly related to glucose concentration in 1- and 2-year-old shoots for non-thinned trees, but only in 2014. In contrast to non-thinned trees, fruit set of thinned trees was not related to glucose concentration. These results were unexpected. Previous research investigating the effects of shading or girdling in addition to anecdotal evidence of the effect of solar irradiance and temperature on fruitlet abscission have highlighted the relationships between factors that influence carbohydrate supply and demand such as photosynthetic rate, competition between fruitlets and actively growing shoots, fruitlet respiration, and fruitlet abscission (Byers et al., 1990a; Palmer et al., 1997; Bepete and Lakso, 1998; Wünsche et al., 2005). Thus, assumption has been that the susceptibility of fruitlets to chemical thinners would be associated with the predicted carbohydrate status of the tree where chemical thinners would more effectively remove fruitlets on trees with low predicted carbohydrate status than trees with high predicted carbohydrate status.

However, the weak relationship observed between fruit set and glucose concentration for thinned trees in this study suggests that the level of thinning was similar regardless of glucose concentration at thinning time. Perhaps measuring other sugars that are more abundant in apple such as sorbitol or sucrose would result in a stronger relationship between fruit set and sugar concentration. However, in wood and fruitlet tissues where soluble sugars were separated, glucose was lower than sorbitol, but sorbitol and glucose concentrations were related and

changed together during the season (McQueen et al., 2004). Additionally, in 2014, fruit set was lower in thinned trees than expected which may have masked the relationship between fruit set and glucose. This seems unlikely, however, as fruit set in 2015 was higher than in 2014 and was also not related to glucose concentration at thinning time.

Chemical thinners such as NAA and carbaryl enhanced fruitlet abscission by suppressing photosynthesis and inducing a carbohydrate stress after application. In addition, fruitlet respiration may also be enhanced following thinner application which exacerbates carbohydrate stress induced by lower rates of photosynthesis (Zhu et al., 2011). Rapid changes in soluble sugar and starch concentrations, gene expression, hormone concentrations, and organic compounds associated with carbohydrate metabolism and transport have been observed in abscised and non-abscised fruitlets (Janssen et al., 2008; Li et al., 2012; Beshir et al., 2017) as well as shoot and pedicel tissues following shading and chemical thinning (Zhu et al., 2011; Zhou et al., 2008). However, to enhance the likelihood of abscission and observe differences in fruitlets, these treatments have been extreme. These studies show rapid genetic and metabolic changes in fruitlets once abscission has been initiated, but again, they provide little evidence for factors that influence the natural susceptibility of fruitlets to chemical thinners.

Reported carbohydrate concentrations of shoots and fruitlets in response to environmental conditions and thinner application has also been mixed (Polomski et al., 1988; Naschitz et al., 2010; Zhu et al., 2011). Perhaps carbohydrate concentration is highly correlated with fruit abscission but the susceptibility of fruit to thinners is affected primarily by environmental stresses such as low light (Byers et al., 1991) or high temperatures (Byers 2002; Atkinson et al.,

2001). The dynamic nature of carbohydrate metabolism and the effects of carbohydrate transport rate or the distance of transport from shoots to fruitlets may also affect fruitlet susceptibility in response to environmental factors and chemical thinners. Consequently, even if carbohydrates are available in the shoot, selective transport from the shoot through the pedicel to the fruitlet may not be rapid enough to support susceptible fruitlets (Yuan and Greene, 2000; Naschitz et al., 2010). Furthermore, concentration gradients of carbohydrate metabolites and cross-talk of hormones such as ethylene and auxin differ in non-abscised fruitlets compared to abscised fruitlets (Janssen et al., 2008; Li et al., 2012; Sawicki et al., 2015). These studies also investigated fruitlet characteristics such as expression of genes involved in sugar metabolism or transport during or after fruitlet abscission; however, the underlying physiology responsible for natural susceptibility of a tree or fruitlet to chemical thinners is still unknown.

In conclusion, mature apple trees have a large capacity to store and metabolize carbohydrates and are robust against environmental stresses or experimental treatments that may affect carbohydrate availability. Our results suggest that fruit set in response to chemical thinners is not directly related to early season glucose or starch concentrations in 1- and 2-year-old wood. The effects of environmental factors and underlying physiological mechanisms that result in fruitlet susceptibility to thinners and abscission should be investigated.

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Table 2.1. Main effect means of 2013 fruit removal treatments (days after full bloom) and chemical thinning on trunk cross-sectional area (TCSA), crop density, total shoot length, and final fruit diameter of mature 'Golden Delicious' apple trees in 2014.

	TCSA (cm^2)	Crop density (fruit/cm^2 TCSA)	Shoot length (cm)	Final fruit diameter (mm)
2013 Fruit removal treatment				
29	122.1a[ZY]	5.79a	33.4a	70.3a
125	119.6a	4.19a	24.8bc	73.5a
154	117.1a	3.22a	28.7ab	75.3a
182	123.8a	4.82a	20.1c	73.8a
Thinning treatment				
Non-thinned	118.0a	6.98a	27.8a	71.3a
Thinned	122.9a	2.04b	26.0a	75.2b

[Z] Main effect lsmeans are adjusted using TCSA measured in 2013 as a covariate.

[Y]Lsmeans for variables within a column followed by common letters do not differ at the 5% level by Tukey-Kramer HSD.

Table 2.2. Effect of rootstock and chemical thinner application on trunk cross-sectional area (TCSA) and crop density of mature 'Golden Delicious' apple trees in 2015.

	TCSA (cm^2)	Crop density (fruit/cm^2 TCSA)	Shoot length (cm)	Final fruit diameter (mm)	Pruning weight (kg)
Rootstock					
M.26	168.5a	2.67a	21.6a	75.9a	9.34a
M.9	140.1a	6.52b	20.5a	73.2a	7.08a
G.16	94.2b	3.86a	15.0b	72.5a	2.14b
Thinning treatment					
Non-thinned	140.4a	6.22a	20.7a	70.8a	6.93a
Thinned	134.2a	2.48b	17.4a	77.2b	5.44a

[Z]Lsmeans for variables within a column followed by common letters do not differ at the 5% level by Tukey-Kramer HSD.

Figure 2.1. Effect of fruit removal (FR) on early season glucose (A and B) and starch (C and D) concentrations of 1- and 2-year-old wood for 'Golden Delicious' apple shoots in 2014. Phenological stages are indicated for dormancy (D), green tip (GT), tight cluster (TC), pink (P), bloom (B), and thinning (T).

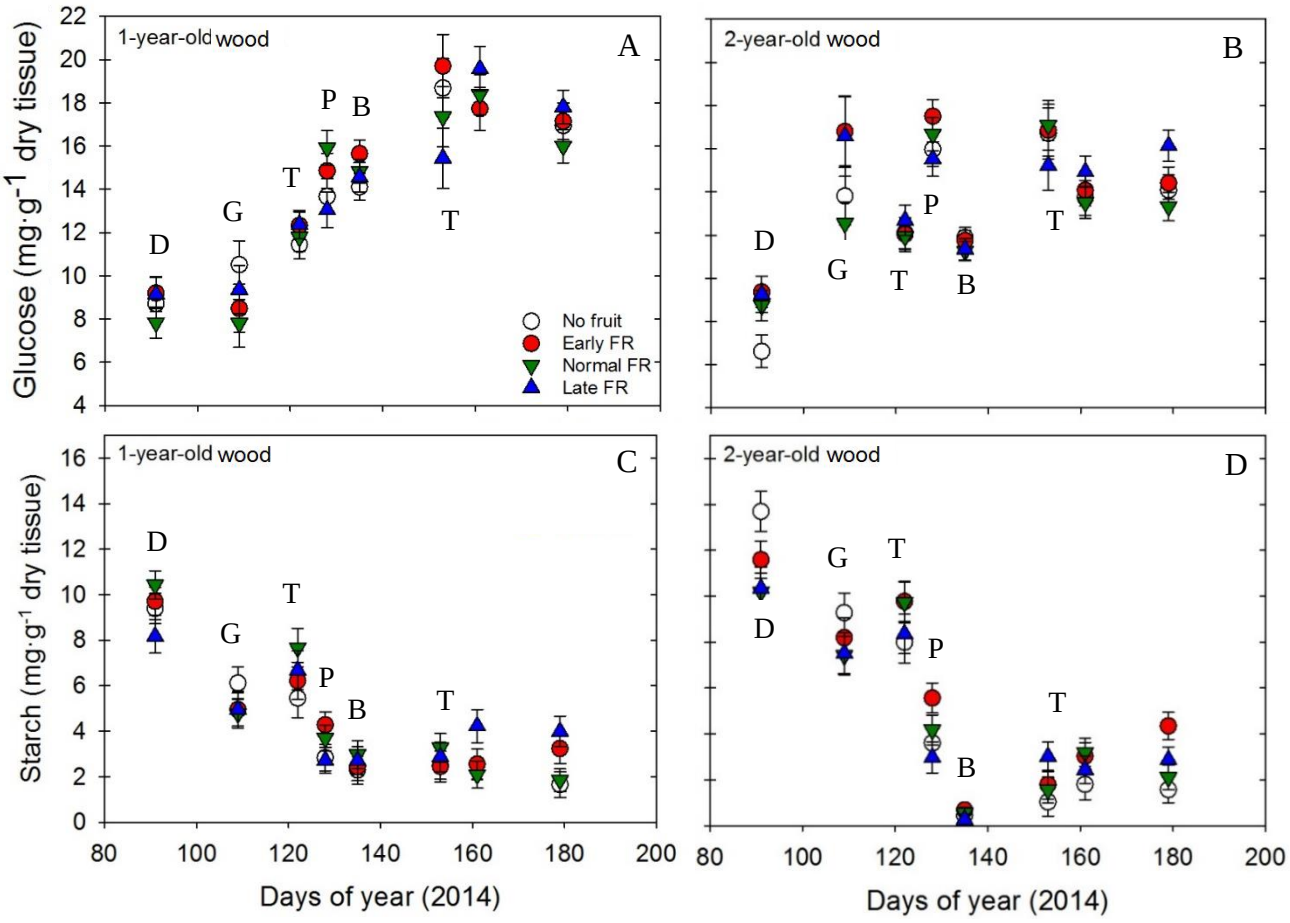


Figure 2.2. The relationship between fruit set (fruit per 100 flower clusters) and glucose (SCC) and starch (SC) concentration at dormancy (A and B) and the day before thinning (C and D) in 2-year-old wood of 'Golden Delicious' shoots for thinned and non-thinned apple trees in 2014. Significance at $P<0.05$ is highlighted in bold, and R^2 values are displayed for each regression.

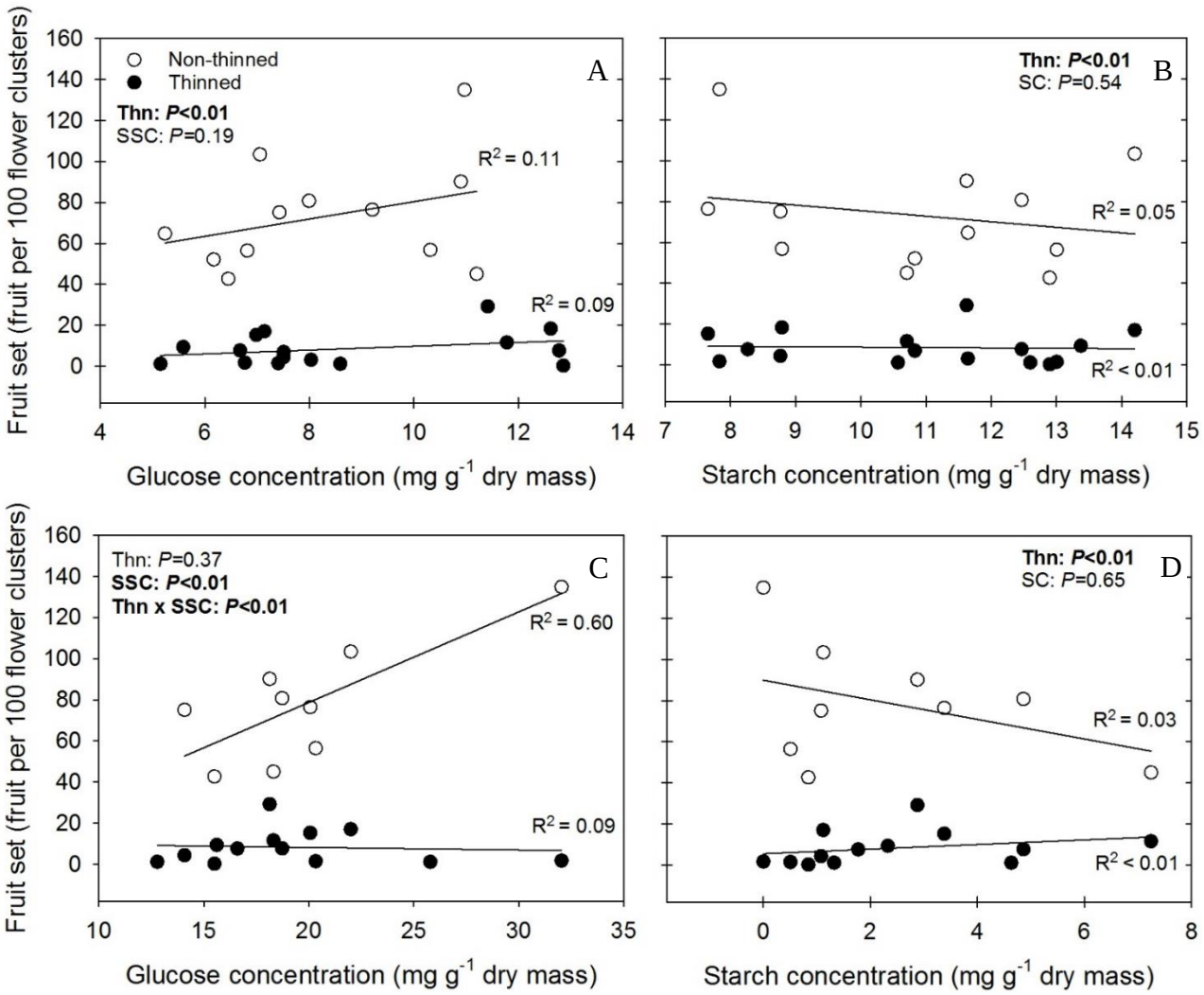


Figure 2.3. The relationship for fruit set (fruit per 100 flower clusters) in 2015 and crop density (CD) in 2014 for non-thinned and thinned (Thn) 'Golden Delicious' apple trees. Significance at $P<0.05$ is highlighted in bold, and R^2 values are displayed for each regression.

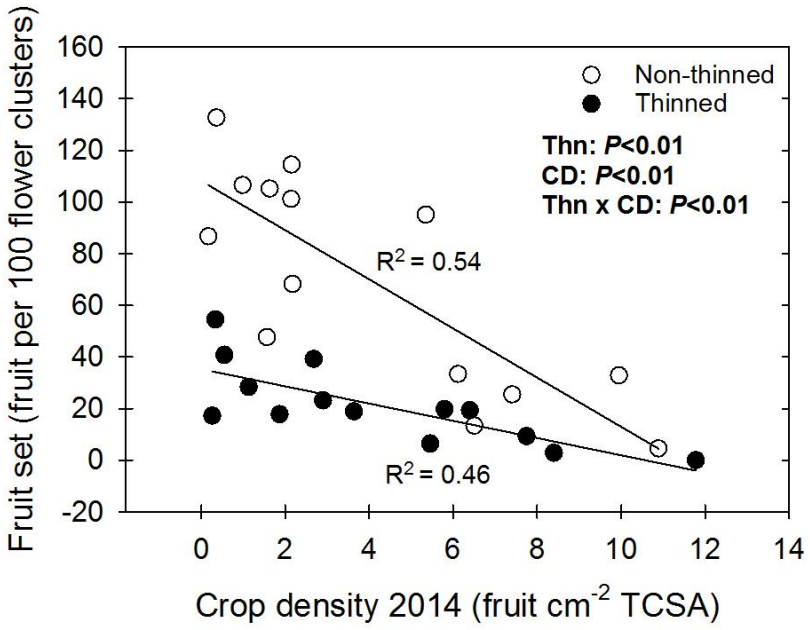
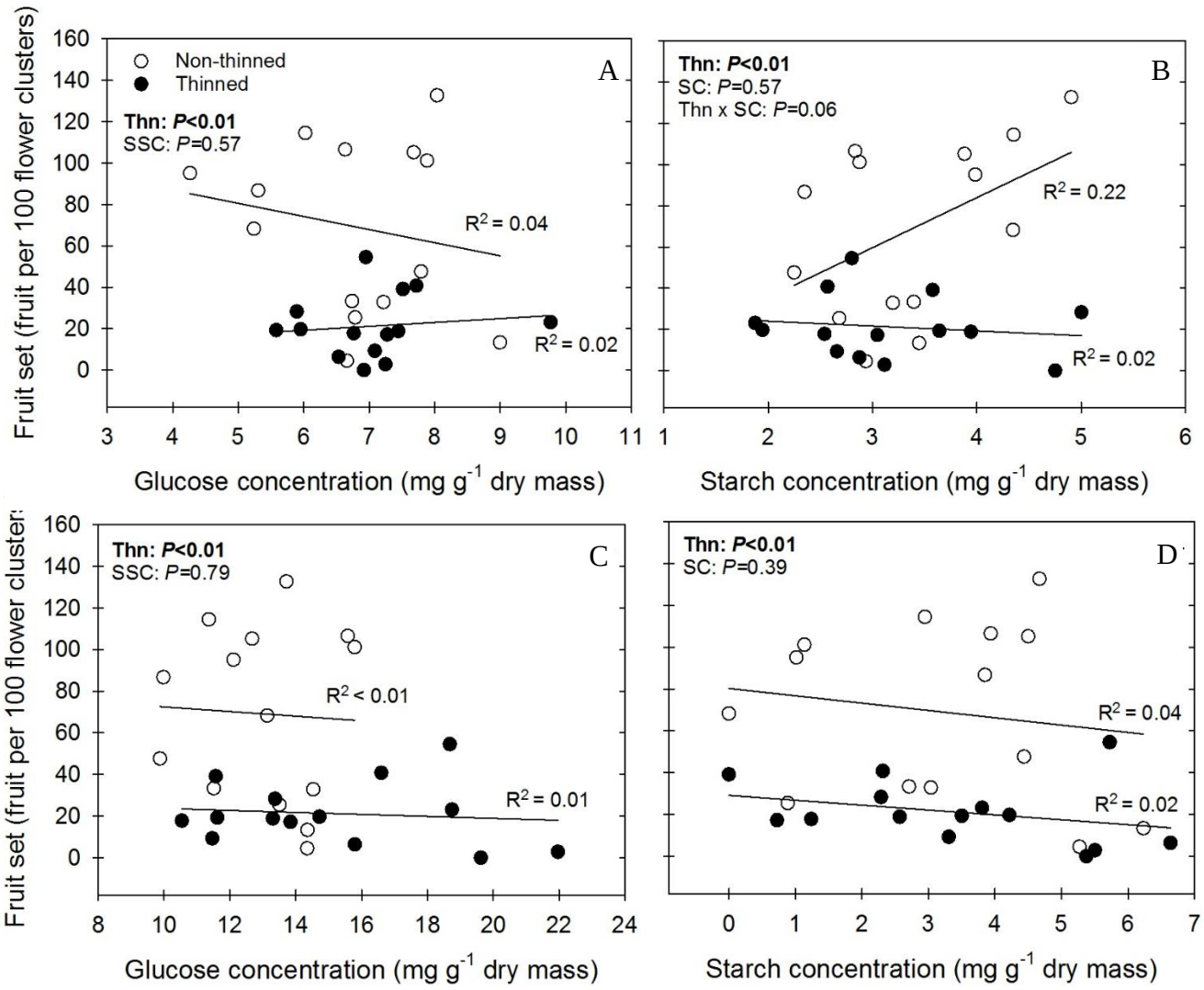


Figure 2.4. The relationship between fruit set (fruit per 100 flower clusters) and glucose (SCC) and starch (SC) concentration at dormancy (A and B) and the day of thinning (C and D) in 2-year-old wood of 'Golden Delicious' shoots for thinned and non-thinned apple trees in 2015. Significance at $P<0.05$ is highlighted in bold, and R^2 values are displayed for each regression.



CHAPTER 3

Root and mycorrhizal fungal foraging responses to fruit removal in apple trees

Abstract. Root and mycorrhizal fungal foraging in nutrient-rich patches is an energy-intensive process, and shifts in carbon (C) availability may affect foraging strategies. We hypothesize that when trees are C limited, they will prioritize root and mycorrhizal hyphal growth in nutrient-rich soil patches. Apple (*Malus* x *domestica* Borkh.) trees with fruit were compared to trees with fruit removed to investigate the effect of reproductive effort and associated shifts in belowground C availability on root and arbuscular mycorrhizal (AM) fungal growth in unfertilized soil and localized nitrogen (N)-rich patches (containing inorganic or organic nitrogen). Across nutrient treatments, fruit removal enhanced root production compared to fruiting trees. In fruiting trees, about four times more roots proliferated in the inorganic-N patch than in unfertilized soil or the organic-N patch. However, in trees with fruit removal, root proliferation was similar among nutrient treatments. Arbuscular mycorrhizal extramatrical-hyphal biomass was not affected by fruit removal but was greater in the organic-N patch than the inorganic-N patch or unfertilized soil. Fruit removal and N addition had modest effects on AM fungal colonization of apple roots and no effect on non-mycorrhizal fungal colonization. Root and AM foraging for nutrients should be considered in the context of C availability. Apple trees may manipulate root foraging more than AM fungal foraging when C belowground is constrained.

Additional index words. arbuscular mycorrhizal (AM) fungi, carbon (C), *Malus* x *domestica*, non-mycorrhizal (NM) fungi, nutrient foraging, nutrient-rich patch

Introduction

Resource foraging is an energy-intensive process, and foraging strategies reflect tradeoffs between the energy cost of foraging and the return of resource capture (Bloom et al., 1985; Charnov, 1976; Hutchings and de Kroon, 1994; Stephens et al., 2007). Plants display multiple strategies for resource capture belowground through enhanced root production (Campbell et al., 1991; Fitter, 1994) and uptake kinetics (BassiriRad and Caldwell, 1992; Jackson and Caldwell, 1992) as well as greater mycorrhizal fungal foraging for nutrients, in some plant species (Chen et al., 2016). However, there is limited evidence connecting plant energy status and environmental cues with root foraging behavior. Furthermore, shifts in plant foraging behavior may impact foraging strategies of mycorrhizal fungi differently from roots as the fungi forage for nutrients in the soil as well as for carbohydrates from plant roots.

As plants manage internal carbon (C) resources at the whole-plant level, they must maintain above-ground growth while also partitioning C belowground to support root interactions with mycorrhizal fungi. Reductions in belowground C availability can occur as a result of aboveground herbivory (Bryant et al., 1983; Kosola et al., 2001), shading (Bilbrough and Caldwell, 1995; Jackson and Caldwell, 1992), canopy scorching, (Guo, et al. 2004) and sizable reproductive effort (Duncan and Eissenstat, 1993). Carbohydrate reductions to roots generally lead to suppressed root growth and occasionally reduced mycorrhizal colonization (Klironomos et al., 2004; Saravesi et al., 2014) but not always (Olsson et al., 2010). Conversely, enhanced C availability belowground, as associated with elevated CO_2 for example, can stimulate root and mycorrhizal fungal productivity (Antoninka et al., 2011; Pregitzer et al., 1995). In 2-yr-old

‘Crispin’ apple trees on the dwarfing rootstock, M.27, fruit removal shifted biomass partitioning and enhanced root dry mass (Palmer, 1992). In this study, we assumed that fruit removal enhanced belowground C availability for root and arbuscular mycorrhizal (AM) fungi although this was not directly measured.

Belowground C availability may also have implications for AM and non-mycorrhizal (NM) fungal interactions. Not only must energy be invested in growing and maintaining root and fungal tissues for nutrient uptake, but additional C is required for defending these tissues from potential herbivores or parasites. Therefore, shifts in C availability can influence NM fungi that colonize roots (Rillig et al., 1998; Runion et al., 1994; Saravesi et al., 2014) which include parasitic, neutral or beneficial fungi. For example, Newsham et al. (1994) reported that AM colonization protected roots of the winter annual grass, *Vulpia ciliata* spp. *ambigua*, from fungal pathogen infection. Thus, stimulation of mycorrhizal fungi by fruit removal may preferentially stimulate AM fungal colonization to the detriment of NM fungi. This could occur through competition by AM fungi for host resources (Linderman, 1994) or enhanced root defenses triggered by AM colonization (Benhamou et al., 1994).

Overlaying this complexity in root and microbial responses to shifts in belowground C is the active foraging of roots and AM hyphae in nutrient “hot spots” in the soil. Both roots and mycorrhizal hyphae readily proliferate in nutrient-rich zones of soil (Chen et al., 2016; Hodge 2004; Pregitzer et al., 1993; through active foraging, or foraging “precision” (*sensu* Campbell et al., 1991). The ability to rapidly deploy nutrient acquisition structures of roots and AM fungi is likely to be influenced by C availability. For example, root production of the bunchgrass,

Agropyron desertorum, was enhanced in N-enriched soil patches compared to unenriched patches. However, when plants were shaded, relative root growth rates were suppressed less in N-enriched nutrient patches than roots in unenriched patches (Bilbrough and Caldwell, 1995).

Active foraging of AM fungi in nutrient-rich patches may be less affected by C limitation; however, other aspects of the mycorrhizae may be altered. Extramatrical hyphae of AM fungi grown in a microcosm proliferated in organic nutrient patches, and although extramatrical hyphal mass was unaffected by shading, root length colonization and arbuscule frequency in the host plant, *Plantago lanceolata* L., was suppressed (Hodge and Fitter, 2010). This supports findings of a container study with *Trifolium subtteraneam* where host plants maintained C supply to AM fungi regardless of shading (Olsson et al., 2010). It also suggests that the nature of the symbiosis may change with shifts in resource allocation from intraradical mycelium to extramatrical foraging structures (Olsson et al., 2010). A limitation of previous studies is that comparisons of root and AM foraging in nutrient-rich patches were conducted in containers with young plants under tightly controlled conditions (Hodge and Fitter, 2010; Johnson et al., 2015; Xu et al., 2008). What happens to root and AM fungal foraging strategies when multiple factors, such as C and nutrient availability, are co-limiting growth in field-grown plants in unconstrained soil? Are roots and AM fungi likely to be more selective in their exploration of the soil when C is limiting than when C is plentiful?

In this study, we examined how fruit removal (reduced reproductive C demand) affected root and mycorrhizal fungal proliferation in nutrient "hot spots" in the soil. We used a high-density apple (*Malus* x *domestica* Borkh.) orchard for this study, as fruit biomass is easily removed in this

system. Under normal production practices, upwards of 80% of the annual biomass in apple is partitioned to fruit, while only 1-2% is partitioned to the roots (Buwalda and Lenz, 1992; Forshey and McKee, 1970; Lakso et al., 1999; Palmer, 1988; Palmer, 1992). In addition, we investigated how fruit removal affected plant interactions with both AM and NM fungi at the individual root level. Previous research of young apple roots (<25 days old) at the location of the present study indicated that AM fungi selectively colonized roots with 20-90% faster growth rates ($mm \cdot d^{-1}$) compared to slower-growing roots. Slower-growing roots were either uncolonized or were colonized by NM fungi (Resendes et al., 2008). This suggests that early colonization by either fungal type may be linked to root C supply although additional factors such as cell wall composition, root exudation, and inoculum density also likely determine colonization events (Brundrett and Kendrick, 1990; Buee et al., 2000; McGonigle and Miller et al., 2000). We hypothesized that (1) root and AM hyphal production would be enhanced by fruit removal and the addition of localized nutrient "hot spots"; (2) root and AM hyphal production would exhibit greater foraging precision (the proportion of total root and fungal hyphal proliferation) in nutrient "hot spots" in trees with fruit (i.e., where belowground C is more limited) than in trees without fruit; (3) NM fungi would colonize a greater proportion of roots when aboveground demand for C was high due to fruit production; and (4) roots with high AM colonization would show limited NM colonization.

Materials and Methods

Field site and experimental design. The field site was located at the Russell E. Larson Agricultural Research Center in Rock Springs, Pennsylvania, USA (40.8°N and 77.9°W, elev.

350 m). The experiment used ‘Golden Delicious’ apple trees on M.9 rootstock, planted in 1997 at 1.8- x 3.1-m spacing and trained on a low-trellis-hedgerow system. Rows were oriented in a north-south direction. Dormant and summer pruning, fruit thinning, and harvesting were done following standard cultural practices used for the low-trellis-hedgerow system and in commercial orchards in the region (Peter, 2018). Soil at the study site was in the Hagerstown series (mesic Typic Hapludalf) with a pH of 7.1 and an organic matter content of 1.7% in the top 0.25 m of the soil profile. Trees were not fertilized for 12 years prior to the experiment.

The experiment consisted of two fruiting treatments (fruit, no fruit) and three localized nitrogen (N) patches (inorganic N, organic N, and unfertilized) with 10 replications each. In Apr. 2013, 80 trees were divided into 20 experimental units. Each experimental unit included four trees where the two inner trees were the experimental trees, and the two outer trees were used as guard trees. One root box (See Appendix; Fig. S1), constructed similarly to those of Comas and Eissenstat (2000), was installed between the two middle trees of each experimental unit within the herbicide strip in Apr. 2013. Dimensions of the root boxes were 70 cm long and wide by 30 cm deep. Root boxes had two viewing windows. One window was on the north side and a second window was on the south side of the root box. Each window faced a different apple tree in the hedgerow system. Viewing windows were further divided into two smaller windows that were 35 cm wide and 30 cm deep (4 smaller windows per box). Smaller windows were made of 3-µm thick sheets of clear acetate film (United States Plastic Corporation; Lima, Ohio) to permit root observation and access for root excision. Soil collected during root box installment was sieved with a 1 mm screen and used to fill in gaps between windows and the undisturbed soil. To minimize temperature fluctuations within the root box, 2.5 cm thick removable Styrofoam® was

placed inside the root box against each window when not in use. A removable lid was also placed over each root box after installation to exclude sunlight and rain.

In May 2013, experimental units were randomly divided into two groups, fruit and no fruit with 10 experimental units in each group. Ten experimental units of the fruit treatment had an average crop load of 40 $t \cdot ha^{-1}$ while young fruit on the remaining 10 experimental units were removed prior to June 4 when fruit were 10-12 mm in diameter. In addition to fruit treatments, each root box received three localized N treatments in separate root box windows. On 31 May when roots first appeared against the observation windows, one of the three N treatments was applied to each window section (only three of four windows were used). For the unfertilized treatment, 20 ml of water were applied weekly in one window section at the soil-acetate film interface during the growing season from 31 May to 25 Oct. 2013. Water was applied across the top of the window at four locations using a pipette. For the organic-N treatment, fully expanded green leaves were randomly collected on 20 May from non-experimental 'Golden Delicious' apple trees. Leaves were dried at 70˚C for five days in a drying oven and ground using a ball mill. At the time of application, four 5 mm soil cores were taken to a depth of 10 cm along the soil-acetate film interface of the treatment window. For each organic treatment window, 5 g of ground leaves were divided and added in the resultant hole from the soil core once on 31 May, 2013. Sieved soil was used to backfilled over the ground leaves to minimize disturbance (See Appendix; Fig. S1B). Following application of ground leaves, 20 ml of water was added weekly during the treatment period. A subsample of the leaves was analyzed for total nutrient content using an elemental analyzer (Vario MAX cube, Elementar; Langenselbold, Germany; Horneck and Miller 1998). Gradual release into soil of about 20% of total N contained in ground leaf

material (2.2% N) was predicted from May to Oct. in 2013 based on regression models of Moore et al. (2006). For the inorganic-N treatment, 20 ml of urea in water at 70 ppm were applied weekly in the third window section to match the concentration of N released by ground apple leaves. Urea was applied across the top of the window at four locations using a pipette. The fourth window section was left untreated and was not used in this study. To verify that cross contamination of localized N treatments across viewing windows did not occur, a dye test was used to observe the diffusion of treatments. Treatments within a treatment window diffused to an area of 18 cm wide and 25 cm deep and did not exceed the dimensions of the treated window.

Soil water content and soil temperature were measured weekly at depths of 15 and 30 cm in each root box using a 10 cm-long time domain reflectometry waveguide read with a TDR 100 (Campbell Scientific Inc.; Logan, UT) connected to a laptop and a HH21 microprocessor-based thermometer (OMEGA Engineering, Inc.; Stamford, CT), respectively. The waveguide and thermometer were inserted horizontally and perpendicular to the treatment windows. Trees in all experimental units were watered weekly by hand from July to Oct. Soil solution was collected weekly in the treatment window sections of 10 root boxes using 5 cm-long micro-lysimeters (Rhizon soil moisture samplers, Eijkelkamp Agrisearch Equipment; Giesbeek, The Netherlands). This solution was sampled 24 h after urea application with 5 ml syringes and stored in vials at -80˚C until analyzed. Samples were analyzed by Brookside Laboratories (New Bremen, OH) for total N concentration. Compared to the unfertilized patch, the localized nutrient addition approximately doubled N availability on average in the soil solution (See Appendix; Fig. S2; P=0.04). Concentrations of N in inorganic and organic nutrient patches were similar. The soil solution N concentration of trees with fruit tended to be higher than that of no-fruit trees

(P=0.06). Neither soil temperature nor soil water content inside the root box were affected by fruit removal (Table S1; P>0.71).

Root observation and sampling. New root growth was traced weekly on the acetate film of the root box windows using a different colored DecoColor® marker (Uchida of America, Corp.; Torrance, CA) for each measurement date. Roots were traced over two observation periods of 31 May to 14 Aug. and 24 Sept. to 25 Oct. 2013. Tracing was more frequent the week before root sampling for each observation period to identify when roots that were 7 days old or younger were actively growing. New growth of these roots was traced the day of sampling and 1, 2, 4, and 6 days before sampling. This approach provided more detailed information on roots at an early age while the total range of root ages was 1 to 70 days old for the first observation period and 1 to 28 days old for the second observation period. On 14 Aug. and 25 Oct., fine roots were sampled and stored in 1.5 ml Eppendorf tubes at 4˚C until further analysis. The traced acetate film used for the treatment windows was removed from the root boxes, cleaned, and scanned at 400 dots per inch (DPI) using an Epson Perfection 4490 Photo Scanner (Epson America, Inc.; Long Beach, CA). From the scanned images of tracings, total root length was measured using the segmented line measurement tool in ImageJ 1.46r (Schneider et al., 2012) for each root in the treatment windows. Total root number for each window was counted, and root age was estimated based on tracing color and sampling date.

Extramatrical and internal fungal development. Soil samples were collected from behind each treated window section in 20 ml scintillation vials. Samples were collected from all root boxes at both root harvest dates. Samples were stored at -20˚C and freeze dried at -5˚C for 4 days. Neutral

lipid fatty acid (NLFA) and phospholipid fatty acid (PLFA) extractions were used to estimate extramatrical hyphae associated with nutrient foraging and spore abundance of AM fungi in the soil behind the treatment windows. Phospholipid and neutral lipid extraction and analysis followed the method described by Bossio et al. (1998). Briefly, neutral lipids and the polar lipids, containing phospholipids, were extracted from 5 g of soil using 4 mL chloroform, 4.5 mL phosphate buffer, and 10 mL methanol. The internal standards for PLFA and NLFA were the fatty acid methyl esters (FAME) 21:0 and 19:0 respectively (Avanti Polar Lipids, Inc.; Alabaster, AL). Lipids were then separated into NLFA and PLFA fractions using silicic acid solid-phase chromatography columns (Thermo Scientific, Waltham, MA). Extracted lipids were fractionated into neutral lipids and polar lipids. Lastly, NLFA and PLFA were converted into fatty acid methyl esters (FAME) through methanolysis. Extracted FAMEs were analyzed using a HP GC-FID (HP6890 series, Agilent Technologies, Inc.; Clara, CA) gas chromatograph. External FAME standards (K101 FAME mix, Grace; Columbia, MD) were used to determine biomass (DeForest et al. 2012). Biomarkers were identified using the Sherlock System (v. 6.1, MIDI, Inc.; Newark, DE).

A subsample of roots of different ages was selected for microscopic observations. Root microscopic quantification of fungal colonization were cleared in 10% KOH at 75˚C. Roots were then stained with 0.05% trypan blue in a lactic acid:glycerol:water (1:1:1) solution for 30 minutes. Roots were destained in a lactic acid:glycerol:water (1:1:1) solution overnight and stored in destain solution until observation (Brundrett et al. 1996). Samples were viewed with a compound light microscope. Individual roots were mounted parallel to the long axis of the microscope slide and covered with a 40x22 mm cover slip. Initially, fungal colonization was

quantified using the traditional approach, and percent of root length colonized was calculated. However, no obvious differences from treatments were detected. Therefore, to examine root and fungal interactions in greater detail, key processes involved in colonization were distinguished: *finding* the root, *entering* the root and *proliferating* inside the root. We used external colonization (% of root length covered with surface hyphae) as an indicator of fungi *finding* the root (determined by hyphal size and growth form). To assess the ability of fungi to *enter* (presence or absence of internal colonization), a subset of the total root population was selected. This subset included only roots that showed external colonization, and no evidence of internal root-to-root colonization was observed. To assess the ability of fungi to *proliferate* in the root (colonization intensity), we measured the percent of root length colonized for the population of roots that showed any evidence of internal colonization. External and internal colonization of both AM and NM fungi were quantified using a modified line-intercept method [McGonigle et al. (1990) as described by Resendes et al. (2008)]. Internal AM fungal colonization was noted when hyphal coils, arbuscules, and/or vesicles were present. Internal NM fungal colonization was noted when cells were filled with septate hyphae and hyphae attached to NM fungal or fungal-like structures (e.g., oospores, zoospores, and sporangia of *Pythium* spp.; zoospores, chlamydospores, and oospores of *Phytophthora* spp.; basidiospores and sclerotia of *Rhizoctonia* spp.; Dugan, 2006). Internal colonization could also arise through internal root-root movement of AM fungal hyphae. However, due to the low levels of internal colonization observed in this study and previous studies showing no evidence of intraradical colonization (Resendes et al., 2008), we suspect that most colonization arose externally.

Statistical analysis. Soil temperature, soil water content, and soil N concentration were analyzed by analysis of variance (ANOVA) using SAS's Mixed procedure (SAS Institute Inc. Cary, NC) where fruit removal was the indicator (class) variable. For soil N concentration, N addition was used as a second indicator variable. Due to variability of root distribution in the soil, only windows containing ≥ five roots were used for the analysis of fungal colonization. The removal of these windows for the fungal colonization analysis was needed to help stabilize (i.e., large potential errors associated with taking a percentile of a small population) colonization estimates within each colonization category. A total of 5 of the 60 windows observed were removed from the analysis. These included two windows of fruit removal with unfertilized and organic-N treatments and three windows of trees with fruit with two windows of mineral-N and one unfertilized treatment. Root box was not a significant source of variation (P=0.20) and treatment windows were analyzed as independent observations. Fruit removal and nutrient addition were indicator variables for root and fungal colonization data. Data of root growth were not normally distributed and were Log_e transformed. Root growth data were analyzed by ANOVA using the Mixed procedure in SAS. Roots that were 7 to 28 days old were used for analysis of external and internal colonization due to limited fungal colonization of roots less than 7 days old (See Appendix; Fig. S3, Fig. S4). External and internal colonization data were arcsin transformed to account for normality prior to analysis by ANOVA using the SAS Mixed procedure. All harvested roots were categorized as uncolonized, only colonized by AM fungi, only colonized by NM fungi, or colonized by both AM and NM fungi. Proportions of roots in each category and the interaction between AM and NM fungi were compared by chi-square analysis using the SAS Freq procedure.

Methodological considerations. In this study, we used fruit removal as a proxy for enhanced C availability belowground. Although nonstructural and stored carbohydrates were not directly measured, previous studies have estimated that upwards of 80% of yearly dry matter is partitioned to apple fruit (Lakso, 1999; Palmer, 1988), and fruit removal normally enhances dry matter production in roots (Palmer, 1988; Palmer, 1992). Fruit removal could also affect root production by altering sink strength for nutrients. For example, individual apple fruit generally contain about 0.2 to 0.3% N and 0.05 to 0.07% P on a dry weight basis (Palmer and Dryden, 2006; Perring, 1964). In contrast, functional leaves generally have 2 to 3% N and 0.1 to 0.25% P. While fruit removal may reduce the relative sink strength for both N and P, often this is compensated by increased aboveground vegetative growth, eliminating any potential decrease in N and P demand aboveground (Maggs, 1963; Palmer, 1992).

Our assessment of root colonization by AM and NM fungi departs from traditional approaches. Generally, internal colonization of a random sub-sample of roots has been used to calculate the percent root length colonized and total fungal root length. However, this approach confounds the ability of fungi to complete three key processes; 1) to *find* the root; 2) to *enter* the root; and 3) to *proliferate* inside the root. In this study, we separated these processes by identifying both external (*find*) and internal (*enter*) colonization as well as colonization intensity (*proliferation*) of individual roots. Thus, we provided greater insight into the complexities of root and fungal interactions of relatively young roots (7-28 days old). Roots of this age are of interest because they are the most active in nutrient uptake (Bouma et al., 2001; Volder et al., 2005).

The quantification of AM fungal biomass using the PLFA and NLFA signature can be a powerful tool to investigate shifts of hyphal biomass in response to localized nutrient patches. One limitation of PLFA and NLFA is that the fatty acid signature for AM fungi may be confounded with bacterial signatures in acidic soils or when background levels of bacteria are high as commonly occurs in agricultural systems (Frostegård et al., 2010; Nichols et al., 1986; Sharma and Buyer, 2015). However, this approach has been shown to be a reasonable relative representation of AM fungal biomass (van Diepen et al., 2007; Olsson, 1999). For example, in six temperate hardwood forests, mycorrhizal colonization and total fungal biomass (PLFA) were enhanced in response to increasing soil pH which was confirmed with molecular techniques (Carrino-Kyker et al., 2016). Although the system used in this study was a hybrid between a forest and savanna (agro-forestry), PLFA and NLFA analyses were coupled to provide a better indication of AM fungal activity. However, NLFA signatures are specific to AM fungal spores and indicate carbon allocation to lipid storage. This was a methodological challenge for our study as we were most interested in abundance of extramatrical hyphae for nutrient foraging. Thus, readers should recognize that references to AM fungal biomass may include some non-AM microbial biomass with PLFA markers.

Results

Effects of fruit removal and nutrient addition on root and mycorrhizal fungal proliferation. There was little evidence that fruit removal enhanced root production during the first observation period from May to Aug. However, fruit removal did enhance root proliferation in unfertilized soil patches by almost three-fold in the second observation period from Sept. to Oct. (P=0.02;

Fruit= 55.6 ± 2.5, No fruit=158 ± 2.7; Fig. 3.1). Roots in the unfertilized zone should reflect root growth patterns of the bulk of the tree roots. Nutrient addition also enhanced root production but only in the second observation period, and this depended on the fruit removal treatment (P<0.01). Surprisingly, there was little evidence that fruit removal or nutrient addition affected AM hyphal biomass associated with the PLFA and NLFA markers at either observation period (P>0.28, Fig. 3.2).

Are root and mycorrhizal fungal proliferation enhanced in nutrient "hot spots" with fruit removal? Trees with fruit prioritized root growth into nutrient-rich patches (particularly inorganic N) (Fig. 3.1) but only in the Sept. to Oct. measurement period when fruit C demand is consistently high (estimated 100 g d^{-1}, according to Lakso et al., 1999). During this time, trees with fruit produced approximately 25% more roots in the inorganic-N patch than in the organic-N patch or unfertilized soil (Fig. 3.1B). Similar trends were reflected in the cumulative root number (See Appendix; Fig. S5) and the total root length (See Appendix; Fig. S6). Fine root production was three times greater in May through Aug. than in Sept. through Oct. Peak fine-root growth occurred in late June and early July, although new roots continued to emerge into late Oct.

In contrast to roots, mycorrhizal fungi did not show a strong interaction between fruit removal and selective proliferation in nutrient "hot" spots. Mycorrhizal fungi produced marginally higher extramatrical hyphae and spore biomass in soil patches with organic-N over inorganic-N (P<0.06; Fig. 3.2), but this was not affected by fruit removal. Lipid (PLFA 16:1ω5c) biomass per individual root was similar across all nutrient treatments in May through Aug. (P=0.25; See

Appendix; Fig. S7). From Sept. to Oct., 54% more AM extramatrical hyphal biomass was produced per root in the organic-N patch compared to the inorganic-N and control patches (P=0.02; See Appendix; Fig. S7); however, these results were heavily influenced by the number of roots produced during each measurement period.

Effects of fruit removal and nutrient patches on mycorrhizal and non-mycorrhizal fungal colonization. We hypothesized that fruit removal would enhance AM fungal colonization and suppress NM fungal colonization. Moreover, assuming that plants are using AM fungi for nutrient foraging, we expected colonization to increase in nutrient "hot spots" as a reflection of increased extramatrical hyphal production. We used traditional approaches to quantify internal colonization on a root length basis and root length production to estimate total AM and NM fungal root length (percent colonization × total root length; See Appendix; Fig. S8). In general, apple roots exhibited relatively low levels of fungal colonization and colonization intensity (See Appendix; Table S2). Averaged over the length of all the absorptive roots, AM and NM internal colonization ranged from 3 to 29%, with no differences among fruiting or nutrient treatments (See Appendix; Fig. S8A, B).

Nutrient treatments affected total AM fungal root length which is the percentage of the total root length colonized by AM fungi. However, this effect depended on whether or not trees were bearing fruit (See Appendix; Fig. S8C; P=0.05) and was heavily influenced by root length production. In trees with fruit, total AM root length was low in control and organic-N patches and high in the inorganic-N patch. In trees with fruit removal, total AM root length was similar across patches and similar in magnitude to the inorganic-N patch of trees with fruit. Total NM

root length was unaffected by fruit removal (P=0.07; See Appendix; Fig. S8D) and nutrient treatments (P=0.10).

When examining the processes of colonization separately: 1) external colonization (*finding* the root), 2) presence or absence of internal colonization (*entering* the root) and 3) colonization intensity (*proliferating* inside the root), key differences among treatments emerged. Only 45% of roots were externally colonized by AM fungi, and neither fruit removal nor localized nutrient addition affected external colonization (P>0.34; Table 1; Fig. 3.3A). This suggests that there was little effect of treatment on the ability of AM fungi to *find* the roots. In contrast, once AM fungi found the root, their ability to enter the root depended on fruit removal and localized nutrient addition. For the subset of roots with external AM fungal colonization, 79% of roots were internally colonized in trees with fruit, but fruit removal reduced the probability that AM fungi entered the root by almost 25% (P=0.01; Fig. 3.3A). Internal colonization was also affected by the type of nutrient patch. The addition of organic N reduced the probability that AM fungi entered the root by about 26% (P=0.03; Table 1).

The ability of AM hyphae to proliferate inside the root was not affected by fruit removal (P=0.21; Fig. 3.3A) but was strongly affected by nutrient source. Of the subset of AM internally colonized roots, the colonization intensity was 70% of the root length in the unfertilized soil. The addition of inorganic or organic-N suppressed colonization intensity by 24% and 47% respectively (P<0.01).

In contrast to shifts in AM fungal colonization, NM fungi was unaffected by nutrient addition or fruit removal. NM fungi externally colonized about 23% more roots than AM fungi (68% across all treatments; Table 3.1), but only 45% of those roots were internally colonized regardless of fruit removal or localized N addition (P>0.33; Table 3.1; Fig. 3.3B). Averaged across all N treatments, internal NM fungal colonization (45%) was about 20% lower than internal AM fungal colonization (65%) for roots showing external colonization (P=0.05). Colonization intensity of NM fungi (57%) was also unaffected by fruit removal or nutrient addition (P>0.23; Fig. 3.3B)

Interactions between AM and NM fungi. We found no evidence that the presence of roots with AM fungi suppressed infection by NM fungi (Table 2). The expected numbers of roots colonized by both AM and NM fungi (i.e., the product of the probabilities of AM-only and NM-only colonization) were similar to the observed numbers (P>0.45; Table 2).

Aboveground growth. For the trees with fruit, final fruit diameter was 70.2 mm ± 3.8. Average fruit weight was 190 g ± 4.0, and average fruit yield per tree was 33.9 kg per tree. Fruit removal did not affect the length of existing individual shoots during the growing season (P>0.66; Fruit= 40.1 cm ± 2.4; No fruit= 39.0 cm ± 2.2; total shoot production was not assessed).

Discussion

We used fruit removal to manipulate C availability belowground for nutrient foraging. Fruiting trees in our study had normal levels of fruit production, which may represent upwards of 60-80%

of total annual biomass production (Lakso et al., 1999; Palmer, 1992). While other aspects of tree physiology may change with fruit removal, including mineral nutrient uptake, leaf metabolic activity, and tree plant growth regulators (Schecter et al., 1994; Wünsche and Ferguson, 2005; Wünsche et al., 2005), the dominant effect is typically increased C availability for vegetative growth both above and belowground (Lakso et al., 1999; Palmer, 1992; Wünsche and Ferguson, 2005). We were specifically interested in how selectivity in root and mycorrhizal fungal proliferation for nutrient foraging may shift with these changes in tree energy status.

Root foraging. In this study, we provide evidence to support our first and second hypotheses when we consider the response of roots to C availability and nutrient addition in localized "hotspots". We found that trees without fruit exhibited a more generalist foraging behavior. Root production was relatively high and non-selective to type of nutrient patch later in the season when C demand from the fruit is highest (Lakso et al., 1999). Alternatively, trees with fruit exhibited an alternative foraging behavior, and roots were considerably more selective in response to localized nutrient "hot spots". This shift in foraging behavior resulted in about three times more root production in the inorganic-N patch than the unfertilized patch for trees with fruit. Thus, selectivity in nutrient foraging needs to be understood in the context of energy status and the relative C available.

The influence of C availability on root foraging precision can be understood from a cost-benefit context. For example, under elevated CO_2, C is generally highly available belowground, and the C costs of construction and maintenance of roots is relatively cheap (i.e., the exchange value of C for nutrients is low, *sensu* Bloom et al., 1985). In this context, nutrients typically limit whole-

plant production so that a larger proportion of total plant-available C is used for root production and maintenance (Bloom et al., 1985). This contrasts to plants grown with limited C available for vegetative growth, such as under low-light conditions or during high reproductive demand. Under these conditions, the value of C is relatively high compared to nutrients so the relative cost of producing a root is high (Bloom et al., 1985).

When C is cheap, roots may use multiple strategies to exploit nutrients in heterogeneous soils such as through enhanced total root system growth (Lukac et al., 2003; Norby et al., 2004), greater mycorrhizal-hyphal production (Norby et al., 1987; Treseder et al., 2003), and enhanced root exudation of plant-derived C in the rhizosphere (e.g., soil "priming" Phillips et al., 2012). Furthermore, non-selective root placement may permit the continual search for nutrients when energy status is high, and C is cheap. Conversely, if energy status is low, plants exhibit alternate foraging strategies. When C is limited, such as from shading (Bilbrough and Caldwell, 1995; Xu et al., 2008) or reproductive effort (this study), plants will deploy roots in locations where there will be the greatest nutrient benefit. Thus, the relative value of the C currency compared to the value of the limiting nutrient currency will partially define the precision of root foraging.

In addition, the source of nutrient may matter for nutrient foraging, as different forms of N may have different costs (Chapin et al., 1987). In this study, the addition of inorganic N stimulated root proliferation more than the patch with organic-N addition in the fruiting trees. The proliferation of roots in the inorganic-N patch was similar regardless of the level of fruit production. Foraging precision was also enhanced with inorganic-N addition compared to organic-N addition, and precision in the inorganic-N patch was further magnified in trees with

fruit. While roots take up both inorganic-N and organic-N forms, they usually preferentially forage for inorganic-N over organic-N forms such as amino acids (Miller et al., 2007; Persson et al., 2003; Xu et al., 2008). Also, nitrate can be a signaling molecule that stimulates root branching and thus, root proliferation (Linkohr et al., 2002). Although in our study we applied urea, the moist, well-aerated soil conditions had the potential for rapid nitrification of the ammonium to nitrate (Schmidt, 1982), thereby potentially stimulating root proliferation directly. Availability of other nutrients, such as P, may also affect root foraging responses. For example, in a subtropical forest, Liu et al. (2015) found that a multi-nutrient addition (nitrogen, phosphorus, and potassium; NPK) stimulated root production more than the addition of N or P alone. Therefore, root proliferation may be context dependent, where the type of nutrient input, the rate of mineralization, and the rate of nitrification could markedly affect the response.

Arbuscular mycorrhizal fungal foraging. In contrast to roots, the responses of AM fungi to fruit removal and nutrient availability were less obvious. There was little evidence to support the first and second hypotheses when only extramatrical hyphal production and total root length colonization were examined. In this study, fruit removal had a marginal effect on AM fungal lipid biomass and total root length colonized. This was unexpected because fruit removal presumably enhanced C availability belowground to both roots and their mycorrhizal fungal counterparts; yet, AM fungi did not respond to shifts in C availability. This is similar to the results of Hodge and Fitter (2010), where shading of *P. lanceolate* had no effect on AM fungal biomass. However, these results differ from other work (Drigo et al., 2008; Rillig et al., 2001; Wearn and Gange, 2007). For example, Drigo et al. (2010) reported that in *Festuca rubra*, elevated CO_2 enhanced lipid biomass of AM extramatrical hyphae (PLFA) and storage organs

(NLFA). Perhaps the relationship between roots and AM fungi is less finely tuned in apple, and AM fungi have little control over C utilization compared to roots. Alternatively, AM fungi may simply forage for carbohydrates from roots regardless of the C status of the host plant where the mycorrhizal fungi may become more parasitic in nature when tree C status is low or when nutrients are abundant (Buwalda and Goh, 1982; Eissenstat et al., 1993; Klironomos, 2003). Mycorrhizal fungi may also scavenge for C from low concentration pools even when the plant is not actively supplying it (Cotton et al., 2010). Therefore, in this study, C limitation may be less influential on hyphal foraging than on root foraging.

There was some evidence that AM lipid biomass was influenced by the type of nutrient patch. The organic-N patch marginally (P=0.06) supported more AM hyphal biomass and sporulation than inorganic-N and unfertilized patches. Enhanced PLFA lipid biomass in the organic-N patch suggests that AM extramatrical hyphae may have selectively foraged for nutrients in the organic-N source over the inorganic-N source and the unfertilized soil independent of whether the trees had fruit or not. Perhaps P in the organic patch stimulated hyphal foraging (See Appendix; Fig. S9). Alternatively, organic molecules may have stimulated mycorrhizal hyphal proliferation (Hodge and Fitter, 2010; Ravnskov et al., 1999). While the mechanism is not clear, temperate forest trees responded similarly, where organic patches preferentially enhanced hyphal proliferation but not root proliferation (Cheng et al., 2016).

Alternatively, AM fungi may simply be a better competitor than roots for N in the organic-N patch compared to the inorganic-N patch regardless of C availability. Mycorrhizal fungi have a high N demand (Hodge, 2000; Hodge and Fitter, 2010), and as fruit removal did not affect

hyphal proliferation, AM fungi may have primarily captured N for fungal growth. If AM fungi are competing with roots in the nutrient patches for N, the benefit of symbiosis for nutrient foraging would be limited (Püschel et al., 2016). Moreover, for a species like apple that can rapidly respond to nutrient-rich patches with root proliferation, there may be greater benefit in root production for readily available inorganic-N sources (NH_4^+ and NO_3^-) that easily diffuse through soil rather than relying on AM fungal foraging in organic-N patches (Johnson et al., 2015). This is consistent with recent findings that temperate trees associated with AM fungi forage more selectively with roots and have little dependence on selective foraging by AM hyphae in nutrient-rich patches (Chen et al., 2016; Cheng et al., 2016).

Effects of fruit removal and nutrient patches on mycorrhizal and non-mycorrhizal fungal colonization. This study highlights the benefits of quantifying AM fungal responses using multiple techniques. Typically, percent root-length colonization is used to quantify internal AM colonization; however, this measurement of AM fungi may not correlate with extramatrical hyphal biomass or spore production (Hart and Reader, 2002). The percentage of the total root-length colonized by AM fungi was lower in apple roots growing in a patch of organic-N than in roots growing in inorganic-N and unfertilized patches in the soil (See Appendix; Fig. S4). In contrast, NLFA and PLFA analysis indicated that AM fungal biomass was higher in the organic-N patch than the inorganic-N and unfertilized patches. Using these complementary techniques provides insight into potential shifts in AM fungal allocation patterns from production of hyphal structures inside the root to extramatrical fungal tissues in response to nutrient source.

Mycorrhizal fungal colonization. Both mineral nutrient and C availability may influence the ability of fungi to forage for carbohydrates derived from the plant roots (Hodge and Fitter 2010; Olsson et al., 2010). There was little evidence that plant C availability or localized nutrient patches affected the ability of AM fungi to *find* roots. In our study, about 41% of roots had the presence of external AM hyphae regardless of treatment. However, when C was constrained in trees with fruit, AM hyphae were 18% more likely to *enter* the root. In addition, there was evidence that the organic-N patch suppressed the ability for AM fungi to *enter* and *proliferate* in the root by about 26% and 47%, respectively. In other words, C availability and type of nutrient patch shifted how AM fungi interacted with roots for each stage of colonization. These shifts may represent the balance between the investment of C available for foraging for roots and internal AM hyphal structures versus that required for mineral nutrient capture by extramatrical hyphae (Gavito and Olsson, 2003; Gavito and Olsson, 2008).

In this study, more than 50% of the apple roots showed no evidence, internally or externally, of AM colonization. Similar colonization rates were also found in grape and peach with 56% and 64% of total root length colonized respectively (unpublished data, Lavely). Low intraradical colonization of these horticulture species may be due to a low reliance on AM fungi for nutrient foraging or soil fertility. Heavily fertilized soils suppress mycorrhizal colonization, particularly soils that are high in P (Biermann and Linderman, 1983; Eissenstat et al., 1993; Johnson et al., 2015). This is not likely the cause of low colonization rates in our fruit trees (See Appendix; Fig. S9). For example, in standard fruit tree production, recommended practices include little to no P or N fertilizer applications although this depends on soil fertility (Peter, 2018). Minimal N availability is recommended to suppress growth of tree structure and encourage C allocation to

fruit. Thus, this production system of limited belowground C availability for nutrient capture and restricted fertilizer inputs should seemingly be an optimal environment for enhanced AM fungal foraging. However, this study provides little evidence that C resources are shifting from roots to AM fungi for nutrient foraging, although the probability that a root would be internally colonized with fruit removal was enhanced.

Non-mycorrhizal fungal colonization. Unlike AM fungi, NM fungal colonization was unresponsive to either C availability or nutrient addition, and we found no evidence to support our hypothesis that NM colonization would be greater in fruiting trees where C availability was limited. While external colonization by NM fungi was high, internal colonization of NM fungi was inhibited regardless of C availability. The fruit-bearing trees in our study may have had sufficient C to restrict internal colonization of potential non-beneficial fungi through physical or chemical defenses (Buchanan et al., 2000; Ellis and Tuner, 2001).

Interactions between mycorrhizal and non-mycorrhizal fungi. There was little evidence to support our hypothesis that AM and NM fungi directly interacted when colonizing roots. We predicted that if AM fungal colonization influenced NM colonization, then the fraction of roots colonized by both types of fungi would be less than what would occur if these two colonization processes were independent (determined by the product of the probabilities of roots colonized by AM and those colonized by NM fungi). The predicted and observed numbers of roots colonized by AM and NM fungi were similar which suggests there was no interaction. Our results differ from previous studies that suggest AM fungi restrict root colonization by NM fungi and fungal pathogens (Newsham et al., 1994; Newsham et al., 1995; Thygesen et al. 2004). Due to the

relatively low level of roots colonized by AM and NM fungi in apple, a larger population of colonized roots would allow for detection of smaller treatment effects.

*Conclusion*s: Plant-available C may directly impact how roots and mycorrhizal fungi interact and forage for nutrients in nutrient-rich "hot spots". Reduced C availability caused plants to exhibit more selective root growth in nutrient "hot spots". Mycorrhizal fungi were less affected by C availability than roots, but reduced C available to roots enhanced the ability of AM fungi to internally colonize the roots. The N source of the nutrient patch affected root proliferation differently from AM fungi, where root production was enhanced by inorganic nutrient "hot spots", and AM extramatrical tissues were possibly enhanced by organic nutrient "hot spots". Collectively, these results suggest that at least for N, plants rely more on root foraging than hyphal foraging to exploit nutrient-rich patches when energy reserves are limited.

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Table 3.1. Arbuscular mycorrhizal (AM) and non-mycorrhizal (NM) fungal colonization of apple roots in response to localized nutrient addition (unfertilized control, inorganic N, and organic N). Roots were 7-28 days old. External colonization represents the percentage of roots exhibiting external colonization by AM or NM fungi. Internal colonization represents the percentage of roots internally colonized by AM or NM fungi of the population that were externally colonized. Colonization intensity represents the percent root length colonized for those roots exhibiting internal colonization.

	AM fungi			NM fungi			*P*-value; difference in colonization between AM and NM fungi
		Roots			Roots		
Treatment	*n*	colonized (%)	*P*-value	*n*	colonized (%)	*P*-value	
External fungal colonization							
Grand mean response		45			68		**0.03**[Y]
Effect of localized nutrient addition							
Unfertilized	68[X]	43 a[Z]	-	57	76 d	-	**0.01**
Inorganic N	129	50 a	-	138	67 d	-	0.12
Organic N	74	41 a	0.60	76	60 d	0.39	0.06

Internal fungal colonization							
Grand mean response		65	-		45	-	**0.05**
Effect of localized nutrient addition							
Unfertilized	25	76 a	-	41	47 d	-	**0.01**
Inorganic N	70	69 a	-	93	46 d	-	**0.03**
Organic N	30	47 b	**0.03**	47	43 d	0.90	0.67

		Root length			Root length		
	n	colonization (%)	*P*-value	*n*	colonization (%)	*P*-value	
Colonization intensity							
Grand mean response		46			58		0.09
Effect of localized nutrient addition							
Unfertilized	25	70 a	-	41	59 d	-	0.99
Inorganic N	70	23 c	-	93	58 d	-	0.23
Organic N	30	46 b	**<0.01**	47	58 d	0.99	0.84

[X]Individual roots were pooled and treated as independent observations because location of root box was not significant (P=0.20). Also shown is the sample size (n).

[Y]P-values (based on χ^2 statistic) of external colonization and internal colonization (when roots were externally colonized) within a particular treatment are shown, and significance at $P \leq 0.05$ is highlighted in bold.

[Z]Different letters illustrate pair-wise comparisons within columns.

Table 3.2. Arbuscular mycorrhizal (AM) and non-mycorrhizal (NM) fungal colonization of apple roots in response to apple fruit removal and localized nutrient addition (unfertilized control, inorganic N, and organic N). Roots were 7-28 days old.

Treatment	Roots internally colonized by AM fungi (%)	Roots internally colonized by NM fungi (%)	AM + NM fungi %		*P*-value; difference in Expected and Observed
			Expected	Observed	
Effect of fruiting					
Fruit	32 (47)[W X]	37 (47)	12[Y]	14 (22)	0.90[Z]
No fruit	26 (42)	23 (38)	6	6 (9)	0.97
Effect of localized nutrient addition					
Unfertilized N	31 (23)	33 (23)	10	14 (13)	0.44
Inorganic N	32 (48)	28 (40)	9	9 (13)	0.92
Organic N	21 (18)	30 (22)	6	1 (5)	0.07

[W]Individual roots were pooled and treated as independent observations because location of root box was not significant (P=0.20). Also

[X]Sample size (n; in parenthesis) is presented for each colonization category.

[Y]Evidence that AM colonization inhibited NM colonization would be reflected in an "Observed" percent colonization of AM + NM fungi to be lower than that of the "Expected", calculated as the product of the probabilities of AM colonized and NM colonized roots.

[Z]P-values (based on χ^2 statistic) within a particular treatment are presented.

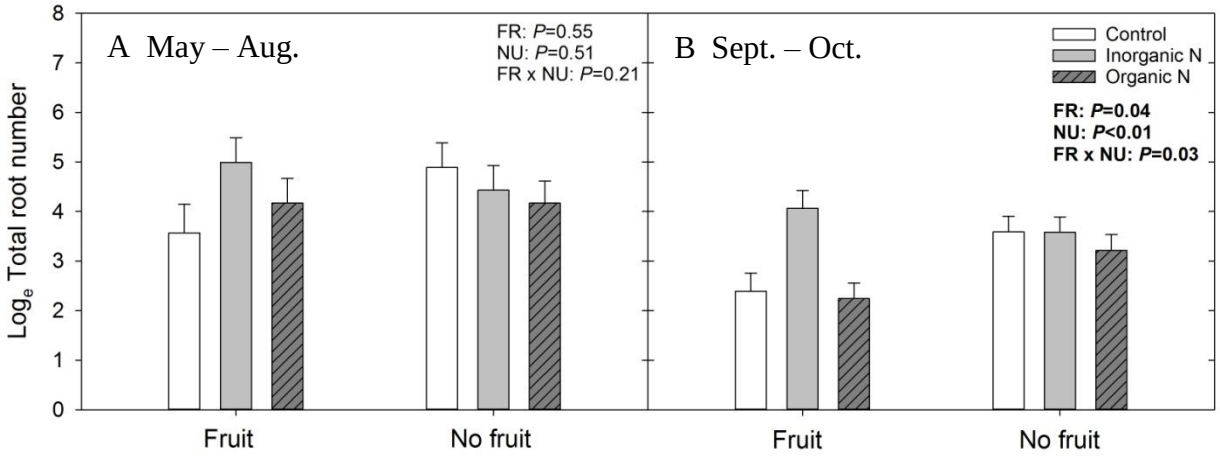

Figure 3.1. Effects of fruit removal (FR) and localized nutrient (NU) addition (inorganic and organic N) on the production of new roots by mature 'Golden Delicious' apple trees on M.9 rootstock. Roots were counted from (A) 31 May to 14 Aug. and (B) 24 Sept. to 25 Oct. 2013. Means are expressed on the Log_e scale, and error bars represent 1 SE. The probability of significance of FR, NU, and the interaction is also shown. Significance at $P \leq 0.05$ is highlighted in bold.

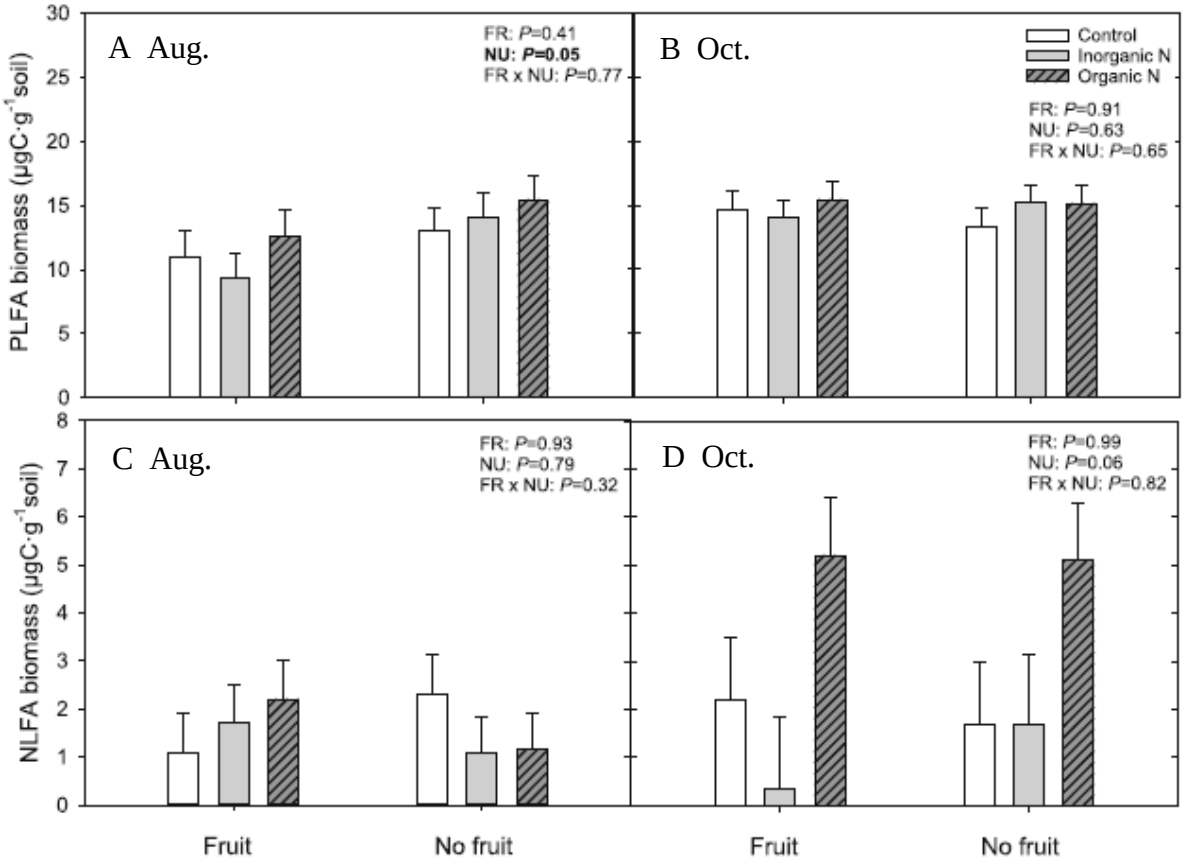

Figure 3.2. Effects of fruit removal (FR) and localized nutrient (NU) addition (inorganic and organic N) on (A, B) phospholipid fatty acid (PLFA) biomass and (C, D) neutral lipid fatty acid (NLFA) biomass of arbuscular mycorrhizal fungi in soil under 'Golden Delicious' apple trees on M.9 rootstock. Samples were collected on (A, C) 14 Aug. and (B, D) 25 Oct. 2013. Error bars represent 1 SE. The probability of significance of FR, NU, and the interaction is also shown. Significance at $P \leq 0.05$ is highlighted in bold.

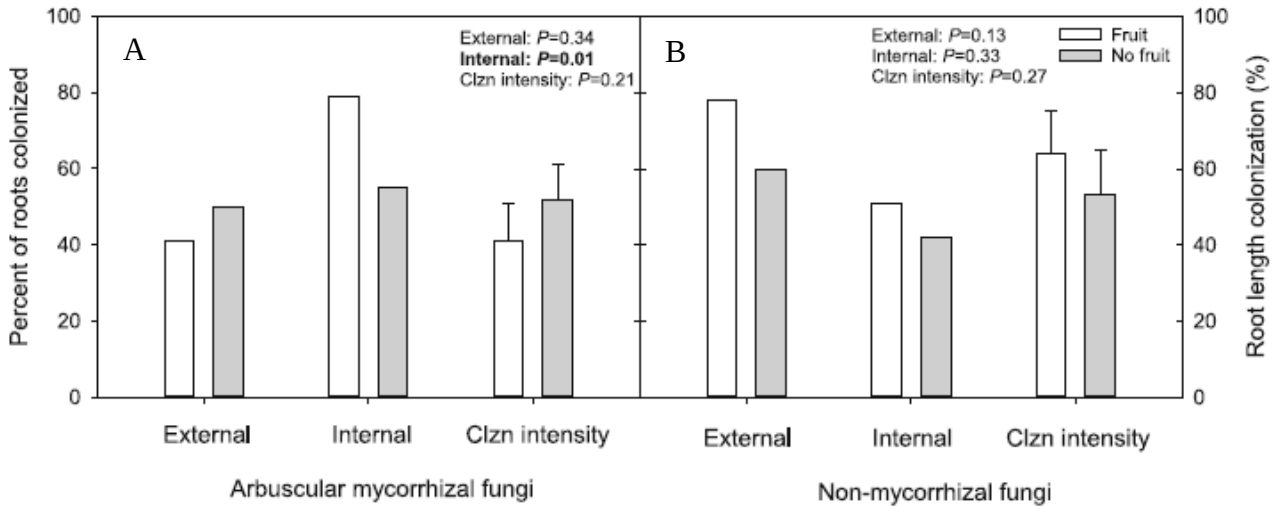

Figure. 3.3. Arbuscular mycorrhizal (AM) and non-mycorrhizal (NM) fungal colonization of roots of 'Golden Delicious' apple trees, on M.9 rootstock, in response to apple fruit removal. External colonization (External) represents the percentage of roots exhibiting external colonization by AM or NM fungi. Internal colonization (Internal) represents the percentage of externally colonized roots that were internally colonized by AM or NM fungi, and colonization intensity represents the percent root length colonized by AM and NM fungi (Clzn intensity) of internally colonized roots. Error bars are not included for external and internal data because percentages were calculated using pooled populations of roots. *P*-values are shown (based on ANOVA). For colonization intensity, errors bars represent 1SE. Significance at $P \leq 0.05$ is highlighted in bold.

CHAPTER 4

On characterizing root function in perennial horticultural crops

Abstract. The fine root system is critical to water and nutrient acquisition, but the characterization of absorptive roots in mature woody plants is challenging. The traditional field approach to fine root classification generally uses an arbitrary diameter cutoff to separate fine from coarse roots, such as the commonly used less than 2 mm. For most woody species, roots less than 2 mm in diameter include both non-woody and woody roots with varying levels of suberization and lifespan. Alternatively, a root order approach can be used to identify absorptive fine roots. We examined the utility of this approach in the horticultural fruit and nut crops: apple (*Malus* x *domestica*), peach (*Prunus persica*), grape (*Vitus vinifera*), almond (*Prunus dulcis*), and citrus (*Citrus* x *clementina*). A second objective was to characterize the variation in first- and second-order roots (most distal) of a wide range of woody horticultural species (33 in total), as diameter variation among species could influence the utility of a diameter cutoff approach, and because diameter has been strongly linked to root function. First-order roots of grape and first- and second-order roots of apple and peach were consistently thin, non-woody, mycorrhizal, and had high N:C ratios. In contrast, fourth- and fifth-order roots of grape and fifth-order roots of apple and peach were woody, non-mycorrhizal, had low N:C ratios, and were thicker than lower order roots. Among the 33 horticultural species, diameter of first- and second-order roots varied about 15-fold, ranging from 0.04 to 0.60 mm and 0.05 to 0.89 mm respectively. The weakness of an arbitrary diameter approach is reflected in comparing, for example, first-order roots of date palm (*Phoenix dactylifera* L.) and lemon (*Citrus x limon* L.), which had diameters as large as

fourth-order roots of apple and peach. Collectively, our research shows that root order characterization has considerably more utility than an arbitrary diameter approach in the identification of roots of different functions in perennial horticultural crops.

Additional index words. fine roots, root diameter, absorptive roots, *Malus* x *domestica*, *Prunus persica*, *Vitus vinifera*

Introduction

Recent advances in root ecology suggest that a root order approach can be more effective than using an arbitrary diameter cutoff, such as less than 2 mm, to characterize roots of different functions (McCormack et al., 2015). In fruit crops, there has also been a tendency to separate ephemeral roots from more structural permanent roots using a diameter cutoff. For example, a study in apple investigating the implications of partial root-zone drying on daily water-uptake rates and root length density of fine roots used a 2-mm diameter cutoff (Green and Clothier, 1999). In addition, seasonal patterns of dry matter accumulation, starch concentration, and N concentration in 'Concord' grapevines were observed in belowground tissues divided into fine roots (< 2 mm diameter), thin roots (2-5 mm diameter), and thick roots (> 5 mm diameter) (Bates et al., 2002). Similarly, the implications of fertilizer application on N, P, and K content and fine root (< 1 cm) growth were investigated in fruiting and non-fruiting pistachio (*Pistachio vera*) trees (Rosecrance et al., 1996). This classification lumps roots of vastly different function in the root system, i.e., the most external, unsuberized, non-woody roots, higher-order suberized roots, and roots that have undergone secondary growth, all in the same category.

A less common approach utilized in research on fruit and nut crops is the root-order approach. This approach is analogous to that used in classification of streams, where the most distal, unbranched roots are 1st order, which are supported by 2nd-order roots and so on (Fitter, 1982; Valenzuela-Estrada et al., 2008; Fig. 4.1). The utility of a root order approach was illustrated in highbush blueberry (*Vaccinium corymbosum*) where 1st- and 2nd-order roots had diameters of only 0.04 - 0.05 µmand exhibited absorptive traits such as high nitrogen to carbon (N:C) ratios, absence of secondary xylem, and abundant mycorrhizal fungal colonization (Valenzuela-Estrada et al., 2008). In contrast, 5th-, 6th-, and 7th-order roots had the largest mean diameters, ranging from 120 to 222 µm, roughly only $1/10^{th}$ that of a 2-mm diameter cutoff. These higher-order roots showed characteristics of transport function such as the presence of secondary xylem, no mycorrhizal fungal colonization, and low N:C ratios. Third- and 4th-order roots were transitional in function. In addition, 1st- to 3rd-order roots were ephemeral and had median lifespans of only 115 to 155 days. Differences in root function based on root order have also been observed in other perennial horticultural crops such as citrus (Eissenstat and Achor, 1999; Rewald et al., 2011) and apple (Wells and Eissenstat, 2001; Wells and Eissenstat, 2003); however, the broad use of root order to investigate root system function has been limited. Can horticultural research be improved in perennial crops by utilizing a root order approach to characterize roots with varying function?

There are also important evolutionary implications to the diameter of 1st-order roots. Previous studies have shown that late-divergent species usually have thinner absorptive roots than species of basal clades (Baylis, 1975; Chen et al., 2013; Ma et al., 2018). However, investigations of root trait diversity across common horticultural crop species are lacking. Selection of beneficial traits

for crop production and fruit quality may have inadvertently modified root traits of horticultural crops compared to their wild-type ancestors. Consequently, the reported phylogenetic conservatism of traits, such as root diameter (Chen et al., 2013; Ma et al., 2018), might be weaker among crop plants.

Here, we studied fine root traits of 33 (in total) perennial horticultural species. One objective of this study was to investigate the utility of using a root-order approach compared to an arbitrary diameter cutoff, such as less than 2 mm, to characterize fine roots of different functions in the horticultural crops: apple, peach, grape, almond, and citrus. We predicted that 1st- and 2nd-order roots would exhibit absorptive traits such as a high N:C ratio, absence of secondary vascular thickening, and mycorrhizal colonization. In contrast, we predicted that 5th-order roots would exhibit transport traits with a low N:C ratio, presence of secondary vascular thickening, and no mycorrhizal colonization while 3rd- and 4th-order roots would be transitional. We also predicted that using a mean root diameter cutoff of 2 mm would include both absorptive and transport roots (up to 5th-order roots) in most horticultural species. A second objective of this study was to characterize the variation in root diameter of 1st- and 2nd-order roots of a wide range of perennial horticultural species and discuss the implications of using a diameter cut-off approach across species. In addition, this survey provides the first compilation of key fine root traits among common horticultural species.

Materials and Methods

Root sampling: Roots of apple and peach were sampled from three established orchards each in Bedford, Berks, and Centre Counties in Pennsylvania (PA). Grape roots were samples from three established vineyards in Lancaster and Union Counties in PA and Frederick County, Virginia (VA). A total of nine trees or vines were samples per species. At each sample location, two root branches were sampled from each of three trees or vines using a hand shovel. Root branches were sampled on opposite sides of each tree or vine and included five root orders (Fig. 4.1). Apple, peach, and grape scions were grafted on Budagovsky.9 (B.9), Lovell, and 101-14 rootstocks respectively. Root samples (three branches per tree) of almond (three trees per variety) were collected from orchards in Yolo (Nonpareil scion on Krymsk 86 (*P. persica x P. cerasifera))* and Glenn (Butte scion on Marianna (*Prunus cerasifera*)) counties in California. For citrus, roots (three branches per tree) were sampled from three trees for a conventional treatment and three trees for an organic treatment at orchards in Tulare county (clementine mandarin) in California. The citrus rootstock was identified as *Citrus sinensis* x *Poncirus trifoliata* (Astrid Volder, personal communication). After sampling, roots were stored in native soil at 4˚C until processing.

Intact root branches were washed with deionized water. Individual root branches from each species were separated into branching order using a modified version of the morphometric approach first developed by Fitter (1982) and described by Pregitzer et al., (2002) and Valenzuela-Estrada et al., (2008). Roots of each order were scanned using a high resolution flat-

bed scanner at 400 dpi (Epson Scanner Perfection 4490; Epson America, Inc.; Long Beach, CA). Roots were dried at 68˚C for 48 hours, and dry mass determined.

Root diameter and total root length were determined from scanned images for each order using WinRHIZO™ (Regent Instruments Inc., need to see which version we have) software (Regent Instruments Inc.; Quebec City, Quebec, Canada). Percent root length (mm) and percent root mass (g) of each order relative to the total of all five orders was calculated for each species, and total root length (m) and root mass (g) of each order were used to calculate the SRL ($m \cdot g^{-1}$). Additionally, we determined for each branching order the total number of roots, branching ratio (the ratio of the number of roots in one order to the number of roots in the next highest order, 1st/2nd, 2nd/3rd, etc.) and branching intensity (the number of roots of one order per unit length (cm) of the next highest order).

To analyze the N:C ratio for roots of each order, roots were pooled across sampling locations. Roots were flash frozen in liquid nitrogen and finely ground with a mortar and pestle. Two 3 mg subsamples were analyzed for total N and total C concentration by flash combustion chromatography (Fisons CHNS-O elemental analyzer, Model EA-1108; Thermo Scientific; Waltham, MA). Soil particle contamination is possible when analyzing C and N concentrations in very fine roots; therefore, N concentration is presented based on C concentration rather than root dry mass.

Microscopy: Arbuscular mycorrhizal (AM) fungal colonization was assessed on a subsample of 10 roots from each root order for each sample location. Roots were cleared in 10% KOH at 75˚C

and transferred to 5% HCl for 5 minutes to enhance clearing. Roots were then stained with 0.05% trypan blue in a lactic acid: glycerol: water solution (1:1:1) for 30 minutes. Roots were transferred to a destaining solution of lactic acid: glycerol: water (1:1:1) overnight and until microscopic observation (Brundett et al., 1996). A compound light microscope was used to view root samples at 600x magnification. Individual roots were mounted on a microscope slide and covered with a 40x22 mm coverslip. Percent AM colonization was quantified using a modified line-intercept method [McGonigle et al., (1990) as described by Resendes et al. (2008)]. Colonization was noted when AM hyphal coils, arbuscules, and/or vesicles were present.

For each species, three roots per order from each site were selected for ultrasections. Roots were stored in ethanol until further processing. Roots were dehydrated through an ethanol series followed by a tert-Butyl alcohol (TBA) series. Following dehydration, roots were embedded in molten Paraplast Plus, a paraffin-polyisobutylene mixture (Sigma-Aldrich Inc., St. Louis, MO). Roots were mounted perpendicularly to the paraffin surface. Once solidified, 10µm-thick cross sections were made using an Ultracut E ultramicrotome (Leica Microsystems, Inc.; Buffalo Grove, IL). Root cross sections were mounted on microscope slides with Haupt solution and dried at 32˚C for 48 hours. Cross sections were then submerged in a series of xylene and ethanol washes to dissolve the paraffin solution. Finally, both safranin O and fast green were used to stain cross-sections as described by Vanden Heuvel and Goffinet (2008). The presence or absence of cork periderm and secondary xylem was noted using a compound light microscope at 100x magnification.

Additional roots were sampled from 33 common perennial horticultural crops, native to tropical, sub-tropical, and temperate regions (Table 1). Two root branches were sampled from each of two trees or plants as described above. Roots were stored at 4˚C until processing. Roots of 1st and 2nd orders were separated, and diameter was measured using a dissecting microscope at 10x magnification.

Statistical analysis. While some traits differed across site ($P<0.05$), we were interested in identifying differences in root order across species despite some site-specific variation. Therefore, mean responses for apple, peach, grape, almond, and citrus were determined with variation among sites included as a source of error. An analysis of variance (ANOVA) was conducted for root diameter, total root length, root number, root mass, percent root length, percent root mass, specific root length, N:C ratio, branching ratio, branching intensity, percent arbuscular mycorrhizal fungal colonization, and secondary vascular thickening with root order (1st through 5th) as the indicator (class) variable of apple, peach, and grape using SAS's Glimmix procedure (SAS Institute Inc., Cary, NC, USA). Similarly, for almond, ANOVA was conducted for root diameter, total root length, root mass, percent root length, percent root mass, and specific root length using root order (1st through 5th) as an indicator variable. For citrus, ANOVA was conducted for root diameter, root length, percent root length, branching ratio, and branching intensity with root order (1st through 4th) as the indicator variable. Multiple comparisons within root order were tested with the SLICE option.

Phylogeny. The phylogenetic tree of all sampled horticultural crop species was drawn in FigTree v.1.4.2 (Rambaut, 2014) based on APG III phylogenetic system. We tested the significance of

phylogenetic signals (Blomberg's K; Blomberg et al., 2003) of the diameter of 1st- and 2nd-order roots using the R function *phylosignal* in the package of *Picante* (The R Project for Statistical Computing, version 3.5.2, www.r-project.org).

Results

Root functional traits. Roots of apple, peach, and grape exhibited both absorptive- and transport-fine root traits in roots of different orders. Across apple, peach, grape, almond, and citrus, root diameter differed by root order where 1st-order roots had the smallest diameter followed by 2nd, 3rd, 4th, and 5th orders respectively ($P<0.01$; Fig. 4.2A). In apple and peach, roots of all 5 orders were less than 2 mm in diameter; in grape, only some 5th order roots were less than 2 mm in diameter. In apple, peach, grape, and citrus, the greatest number of roots on a root branch were of the 1st order followed by 2nd, 3rd, 4th, and 5th orders respectively ($P<0.01$; Table 1). More total root length was observed in lower- (1st, 2nd, and 3rd) than higher- (4th and 5th) order roots for all five species ($P<0.01$; Table 1). In contrast to total root length, total root mass was lower in 1st, 2nd, and 3rd root orders than 4th and 5th root orders for apple, peach, and grape ($P<0.01$). The specific root length ($m \cdot g^{-1}$) was higher in 1st-, 2nd-, and 3rd-order roots than 4th- and 5th-order roots in apple, peach, grape, and almond ($P<0.01$; Fig 4.2B). In other words, there was more length and absorptive surface area per unit of mass for lower- than higher-order roots. Additionally, N:C ratio was higher in 1st-, 2nd-, 3rd-order roots for apple and peach and 1st- and 2nd-order roots for grape than higher- (up to 5th) order roots ($P<0.01$; Fig. 4.2C).

Percent root length differed by root order regardless of species, and about 60% of the root length on a root branch was in the first three root orders (P<0.01; Fig. 4.3A).

Also, percent root mass was lowest in 1st-, 2nd-, and 3rd-order roots while 4th-order roots made up about 25% of the root mass and 5th-order roots comprised about 50% of the root mass (P<0.01; Fig. 4.3B). The branching ratio, or the ratio of the number of roots in one order to the number of roots in the next highest order, differed across branching levels of apple, grape (P<0.01; Table 1) and citrus (P=0.05), but peach had similar branching ratios regardless of order (P=0.42). For apple, 3rd/4th order roots had the highest branching ratio of 4.04 while 1st/2nd order roots had the lowest branching order ratio. In grape, 2nd/3rd roots had the highest branching ratio while 4th/5th order roots had the lowest. Branching ratio of citrus was markedly different where the ratio of 2nd/3rd order roots (9.70) was higher than 1st/2nd order roots (3.54) and 3rd/4th order roots (3.92; P=0.05). Branching intensity, or the number of roots of one order per unit length (cm) of root of the next highest order, decreased with increasing root order from 5.27 to 0.49 for apple, 6.26 to 0.54 for peach, and 2.48 to 0.31 for grape (P<0.01; Table 1); however, for citrus, the branching intensity was higher for 2nd/3rd order roots (0.57) than 3rd/4th order roots (0.15) and 1st/2nd order roots (0.25; P<0.01).

The percent root length colonized by AM fungi was higher in 1st-, 2nd-, and 3rd-order roots of apple, peach, and grape (P<0.01; Fig. 4.4A). Fourth-order roots had low levels of colonization, and 5th-order roots had no colonization. In contrast to mycorrhizal colonization, secondary vascular thickening was absent in 1st-, 2nd-, and 3rd-order roots of apple, 1st- and 2nd-order roots of peach, and 1st-order roots of grape (P<0.01; Fig. 4.4B). The percentage of roots with

secondary vascular thickening increased with root order, and all 5th-order roots of apple and peach, and 4th- and 5th-order roots of grape had secondary vascular thickening.

Phylogeny. Across the 33 horticultural species, diameter of 1st- and 2nd-order roots varied greatly (Fig. 4.5) and ranged from 0.04 to 0.60 mm and 0.05 to 0.89 mm respectively ($P<0.01$; Table 4.2). We found a significant signal of phylogeny in both 1st-order (Blomberg's K = 0.94, $P=0.01$) and 2nd-order root diameter (Blomberg's K = 1.10, $P<0.01$), indicating that order-based root diameters are phylogenetically conserved across the studied horticultural crop species (i.e., explained by phylogenetic relatedness; Fig. 4.6). In addition, the phylogenetic signal of root diameter was apparently stronger in 2nd-order roots than 1st-order roots. Basal lineages, such as *Citrus* species, had thicker roots than lineages that diverged more recently, such as *Prunus* species.

Discussion

In this study, a root-order approach proved useful at distinguishing primarily absorptive roots from those with primarily transport functions in the species examined. First-, 2nd-, and 3rd-order roots of apple; 1st- and 2nd-order roots of peach; and 1st-order roots of grape exhibited absorptive functional traits such as a higher N:C ratio, arbuscular mycorrhizal colonization, and absence of secondary vascular thickening, which is consistent with other studies (Eissenstat and Achor, 1999; Pregitzer et al., 2002). Fourth-order roots of apple, 3rd- and 4th-order roots of peach, and 2nd- and 3rd-order roots of grape were transitional in that some roots had a mix of absorptive functions such as mycorrhizal colonization and transport functions like secondary

vascular development. At the level of 5th-order in apple and peach and 4th-order roots in grape, all roots exhibited traits with primarily transport functions such as a low N:C ratio, no arbuscular mycorrhizal colonization, and secondary vascular development. Although indicators of absorptive capacity differed between lower- and higher-order roots, mean root diameter of all five root orders was less than 2 mm for apple, peach, and grape. Mean diameters of 5th-order almond roots and 4th-order citrus roots were also less than 2 mm.

The first, two, root orders of apple, grape, peach, almond, and citrus had the highest SRL and branching intensity and largest percent root length compared to higher-order roots. Lower-order roots may account for upwards of 75% of the total root length in the first, five, root orders (this study; Guo et al., 2004) and they respond readily to environmental cues through enhanced root production and uptake capacity. For example, irrigation (Comas et al., 2005) and inorganic-N (Guo et al., 2004; Lavely et al., 2018) can stimulate absorptive root production. In addition, N uptake is most rapid in young, 1st-order, roots (Volder et al., 2005).

Our results are similar to non-horticultural studies that have used the root-order approach to investigate fine root function in woody forest species. For example, in temperate trees of North America and China, morphological differences were observed in roots of different orders. First-order roots had the smallest diameters, the largest proportion of total root length, the highest specific root length (SRL), and the highest N concentration compared to higher order roots (Guo et al., 2008a; Guo et al., 2008b; Pregitzer et al., 2002; Wang et al., 2006). This has been a valuable approach for the characterization of fine root architecture, morphology, and function and has led to a greater understanding of belowground processes in forest ecosystems (Guo et al.,

2004; Guo et al., 2008b; McCormack et al., 2015; Pregitzer et al., 2002). The same appears to be true for perennial horticultural systems.

In previous research, root diameter has been a helpful proxy for root function and is often correlated with other plant traits related to physiology, morphology, and function. Root diameter has also been used to describe broader ecological processes such as nutrient foraging, fine root turnover, and nutrient cycling in forest systems (Chen et al., 2016; Eissenstat et al., 2015; McCormack et al., 2012). For example, variation in 1st-order root diameter across species is positively correlated with root life span and mycorrhizal fungal colonization and negatively correlated with opportunistic root foraging (Comas and Eissenstat, 2009; Eissenstat et al., 2015; Ma et al., 2018; McCormack et al., 2012). In these studies, general patterns emerged that linked root diameter (thickness) to other root traits, function, and mycorrhizal associations. With the use of a more effective approach to distinguish the functional traits of fine roots, such as root order, similar advances may be revealed in horticultural plants.

If an arbitrary diameter approach, such as using a 2-mm diameter cutoff (e.g., Bates et al., 2002; Green and Clothier, 1999), had been used to classify fine roots with absorptive function for apple in our study for example, only about 57% of roots included would have been non-woody roots. Similarly, for peach, about 64% of roots included would have been non-woody, and for grape, only 53% of roots would have been non-woody. In addition, if only fine root mass was determined, inclusion of woody roots (4th and 5th orders) would have represented about 65-76% of the total fine root mass, illustrating the large errors in estimating the non-woody, absorptive roots by an arbitrary diameter cutoff approach. While these higher-order, woody roots are likely

long-lived, the lower-order, ephemeral, roots may have lifespans as short as a few weeks (Wells and Eissenstat, 2001). Therefore, using a 2-mm diameter cutoff to characterize ephemeral fine roots can lead to substantial errors.

Furthermore, there is growing evidence that variation across species in root morphology is vast, even exceeding variability in leaf thickness (Ma et al., 2018, Wright et al., 2004). Diameters of 1st- and 2nd- order roots observed in the 33 species in this study ranged about 15-fold in root diameter across different plant families. When comparing roots of different species, it is difficult to use a single diameter cutoff across species to capture root function. For example, the absorptive fine roots of apple include the first three root orders. A diameter cutoff could be used where roots with diameters of ≤ 0.3 mm would be classified as absorptive fine roots, and roots with diameters > 0.3 mm would be considered transport fine roots. However, if we applied that same diameter cutoff of 0.3 mm to a thick-rooted species like citrus, almost all roots would be excluded.

Separating roots into individual orders is time consuming. To enhance processing time, separating roots into functional classes of absorptive fine roots and transport fine roots may be useful option. This could be achieved by grouping roots based on root order. For example, to characterize the proportion of the root system that is primarily responsible for water and nutrient uptake in apple, 1st- through 3rd-order roots may be combined as absorptive fine roots and separated from higher-order roots. Alternatively, use of a diameter cutoff within a species, as mentioned above with apple (0.3 mm), may effectively differentiate absorptive and transport fine

roots; however, a unique diameter cutoff should be determined for each species to make functional comparisons.

An additional consideration is the difference between lower-order ephemeral roots and pioneer roots. In contrast to thinner, ephemeral roots that are intended, primarily for water and nutrient absorption, pioneer roots are designed to become the structural framework of the root system. Pioneer roots have different characteristics than absorptive fine roots which may include thicker diameter, lower specific root length (m g^{-1}), higher growth rate (mm d^{-1}), and no mycorrhizal colonization (Zadworny and Eissenstat, 2011). When first formed, pioneer roots are also 1st-order roots; however, because of their structural and functional differences compared to absorptive fine roots, it is important to distinguish these root types when characterizing fine root function.

In conclusion, using an arbitrary diameter cutoff, such as 2 mm, to classify fine roots in the horticultural crops, apple, peach, and grape, would have led to erroneous estimates of absorptive fine roots by including both woody and non-woody roots. The differences observed in lower-order, absorptive versus higher-order, transport fine roots in the species examined highlight the potential utility of characterizing fine roots using a root-order approach in perennial horticultural crops. Moreover, perennial horticultural species vary widely in root diameter, limiting a single diameter approach for cross-species comparisons.

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Table 4.1. Least squares means of root morphological and architectural traits of five root orders for apple, peach and grape across sampling locations.

Species	Root order	Root number	Total root length (mm)	Root mass (g)	Root order ratio	Branching ratio	Branching intensity
Apple							
	1	180	320	0.0079	---	---	---
	2	81.6	337	0.0077	1st/2nd	2.19	5.27
	3	26.9	325	0.0108	2nd/3rd	3.64	2.83
	4	8.22	227	0.0190	3rd/4th	4.04	1.44
	5	2.89	188	0.0406	4th/5th	3.08	0.491
P-value: Order		<0.01	<0.01	<0.01	---	<0.01	<0.01
Peach							
	1	247	556	0.0103	---	---	---
	2	79.5	429	0.0086	1st/2nd	3.76	6.26
	3	33.9	473	0.0202	2nd/3rd	2.62	1.79
	4	11.7	399	0.0471	3rd/4th	3.93	0.931
	5	3.61	292	0.1054	4th/5th	3.83	0.535
P-value: Order		<0.01	<0.01	<0.01	---	0.42	<0.01

Grape							
	1	71.2	356	0.0099	---	---	---
	2	24.2	324	0.0226	1st/2nd	3.13	2.48
	3	7.11	265	0.0338	2nd/3rd	4.18	1.12
	4	3.67	202	0.0702	3rd/4th	2.46	0.314
	5	1.89	152	0.1362	4th/5th	2.02	0.310
P-value: Order		<0.01	<0.01	<0.01	---	<0.01	<0.01

Table 4.2. Least squares means for root diameter of 1st and 2nd order roots and reported arbuscular mycorrhizal (AM), ectomycorrhizal (EM) and ericoid mycorrhizal fungal associations for 33 common perennial horticulture crops.

		Root diameter		Mycorrhizal
Scientific name	Common name	1st order	2nd order	association
Agave americana L.	agave	0.46	0.60	AM
Prunus dulcis	almond	0.14	0.19	AM
Malus x *domestica*	apple	0.19	0.21	AM
Pyrus pyrifolia L.	Asian pear	0.27	0.29	AM
Persea americana Mill.	avocado	0.52	0.80	AM
Musa sp. L.	banana	0.26	0.39	AM
Ribes rubrum L.	black currant	0.19	0.26	AM
Sambucus nigra L. spp. *canadensis*	black elderberry	0.52	0.64	AM
Vaccinium corymbosum L.	blueberry[Z]	0.04	0.05	Ericoid
Theobroma cacao L.	cacao	0.19	0.21	AM
Cocos nucifera L	coconut	0.29	0.89	AM
Coffea arabica L.	coffee	0.30	0.45	AM
Ficus carica L.	common fig	0.14	0.20	AM

Diospyros virginiana L.	American persimmon	0.57	0.63	AM
Phoenix dactylifera L.	date palms	0.60	0.70	AM
Punica granatum L. 'Nana'	dwarf pomegranate	0.29	0.34	AM
Ribes uva-crispa L.	gooseberry	0.09	0.14	AM
Vitis vinifera L. (hybrid)	grape	0.27	0.33	AM
Psidium L.	guava	0.30	0.38	AM
Citrus x *limon* L.	lemon	0.60	0.70	AM
Litchi chinensis	lychee	0.08	0.12	AM
x *Citrofortunella* 'Indio'	mandarinquat	0.46	0.54	AM
Citrus x *meyeri*	Meyer lemon	0.52	0.65	AM
Olea europaea L.	olive	0.45	0.54	AM
Asimina triloba L. Dunal	pawpaw	0.55	0.74	AM
Prunus persica L.	peach	0.15	0.19	AM
Ananas comosus L. Merr	pineapple	0.13	0.16	AM
Prunus domestica L.	plum	0.26	0.27	AM
Rubus idaeus L.	raspberry	0.19	0.23	AM
Sassafras albidum	sassafras	0.59	0.69	AM
Prunus avium L.	sweet cherry	0.39	0.40	AM

Juglans nigra L.	walnut[Y]	0.38	0.42	AM

All species form associations with arbuscular mycorrhizal fungi except for blueberry which forms associations with ericoid mycorrhizal fungi. Black elderberry may form mycorrhizal associations with both arbuscular and ectomycorrhizal fungi.

[Z] Values of blueberry are reported from Valenzuela-Estrada et al., 2008.

[Y] Values of walnut are reported from Eissenstat et al., 2015.

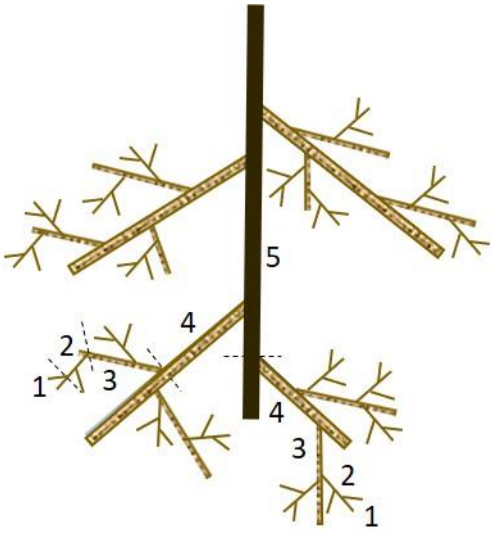

Figure 4.1. A schematic diagram of a *Prunus persica* root branch which includes five root orders. Black dotted lines represent the regions of that specific root order as labeled by numbers. Line thickness represents the mean root diameter of that order. Color represents absorptive (light brown), mottled (transition stage), and transport (dark brown) root functions.

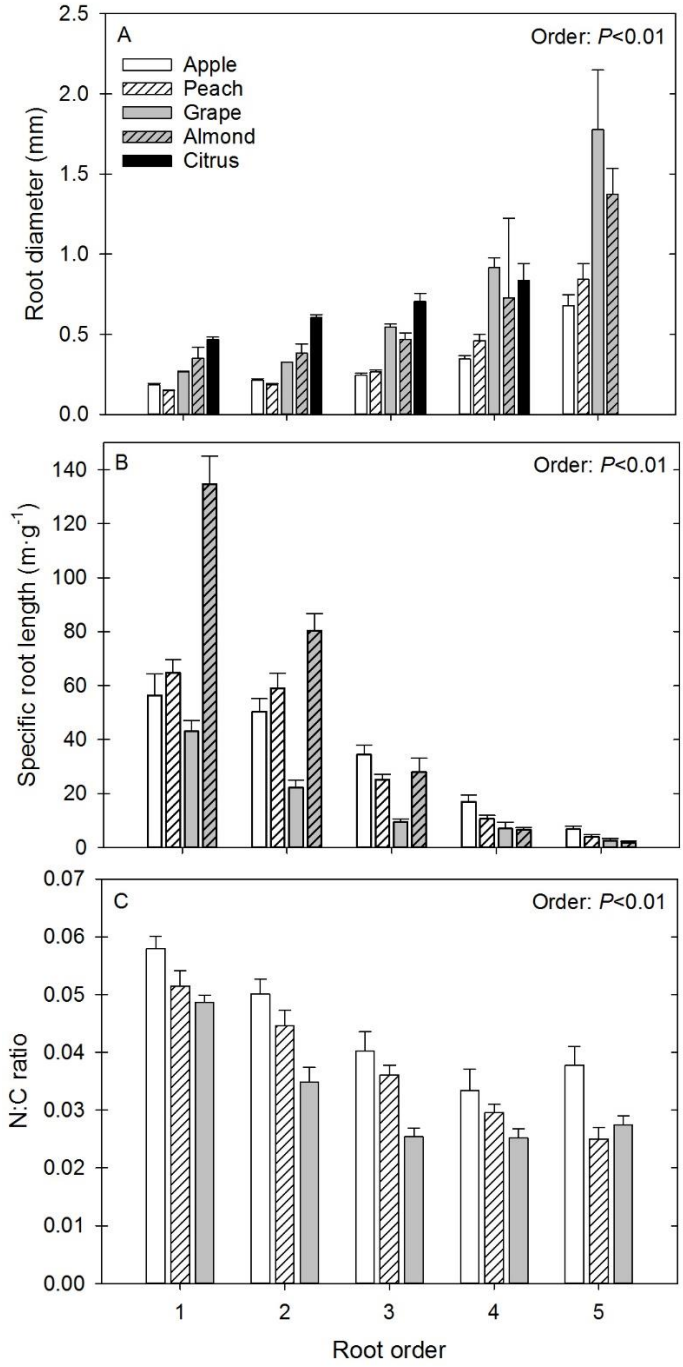

Figure 4.2. Mean root diameter (A), specific root length (B), and N:C ratio (C) of up to five root orders of apple, peach, grape, almond (N:C ratio not shown), and citrus (specific root length and N:C ratio not shown) are presented across all sampling locations. Error bars represent 1 SE, and significance of root order (Order) is presented based on ANOVA.

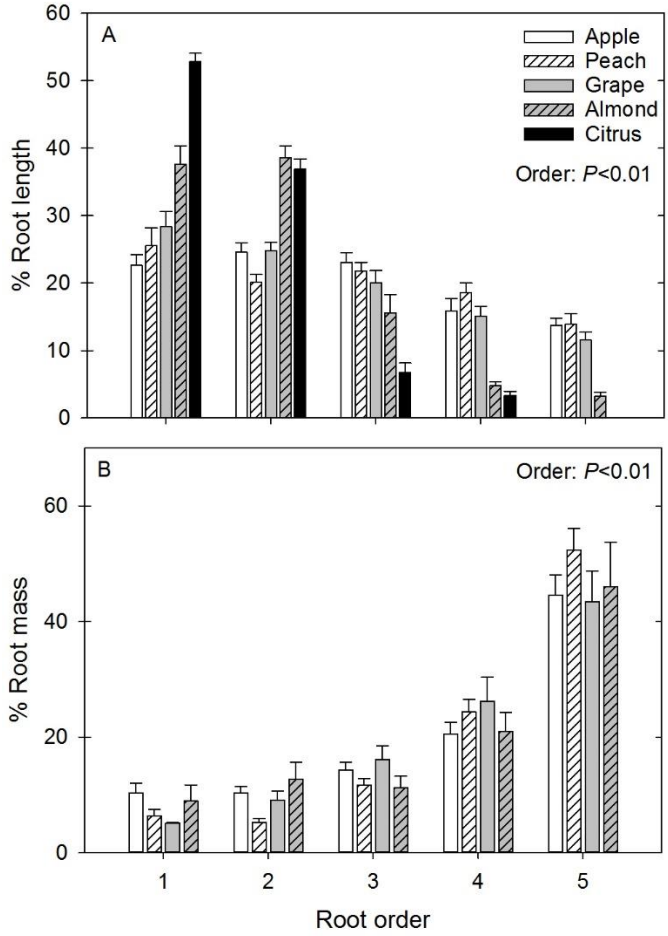

Figure 4.3. Mean percent root length (A) and percent root mass (B) of 1st, 2nd, 3rd, 4th, and 5th root orders for apple, peach, grape, almond, and citrus (percent root length only up to the 4th order) across all sampling locations. Percentages were determined from the proportion of roots of each order from representative root branches of the three crops (n=18). Error bars represent 1 SE, and significance of root order (Order) is presented (based on ANOVA).

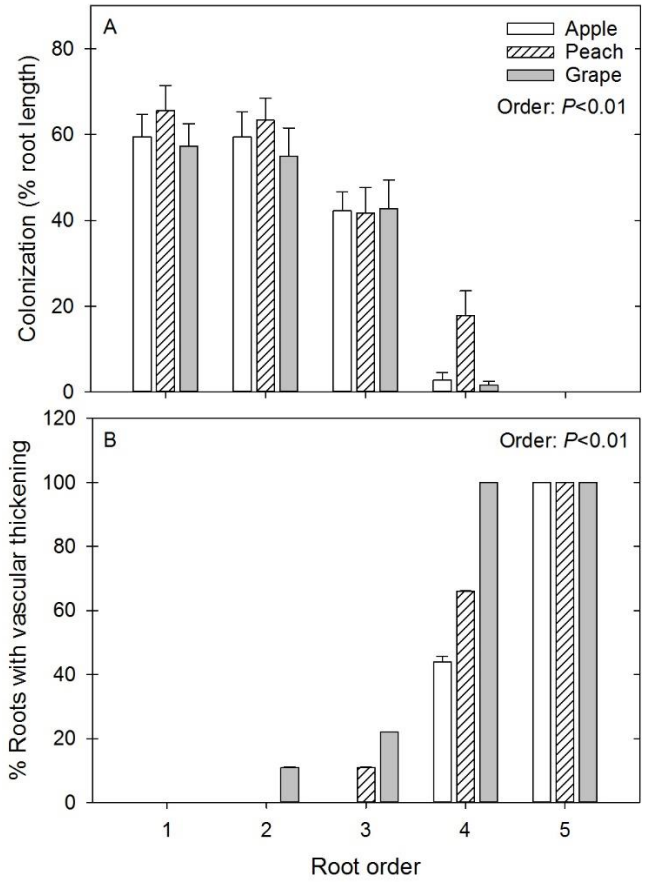

Figure 4.4. Mean percent root length colonized by arbuscular mycorrhizal fungi (A) and percent of roots with vascular thickening (B) of 1st, 2nd, 3rd, 4th, and 5th root orders for apple, peach, and grape across all sampling locations. Error bars represent 1 SE. If all roots had vascular thickening, no error bars are present. Significance of root order (Order) is presented (based on ANOVA).

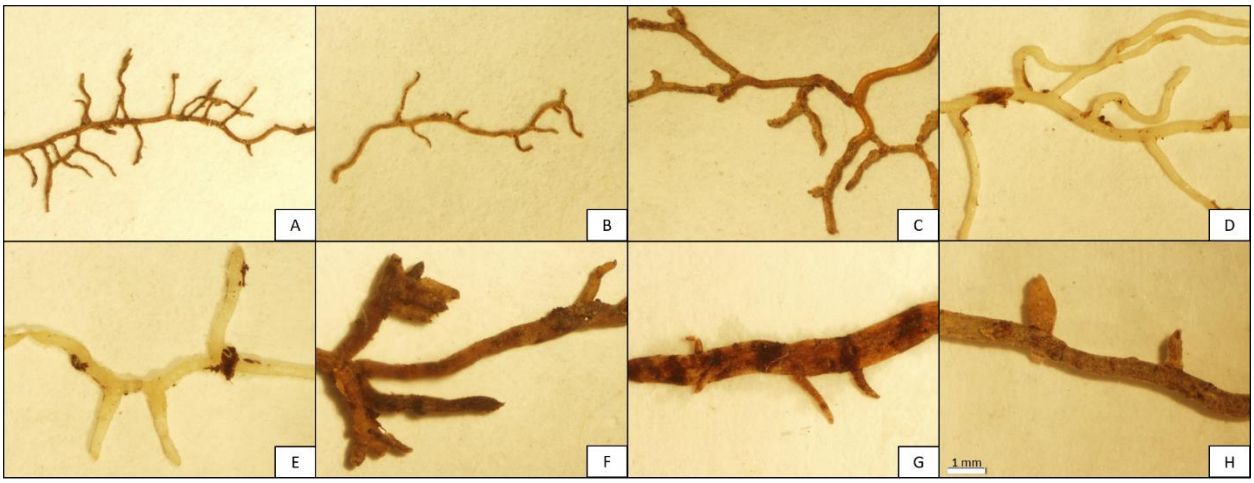

Figure 4.5. Micrographs showing differences in fine roots of eight common horticultural crops. Roots of (A) gooseberry (*Ribes uva-crispa*), (B) fig (*Ficus carica*), (C) apple (*Malus* x *domestica*), (D) coffee (*Coffea arabica*), (E) agave (*Agave americana*), (F) olive (*Olea europaea*), (G) coconut (*Cocos nucifera*), and (H) date (*Phoenix dactylifera*) are shown. Scale bar represents 1 mm.

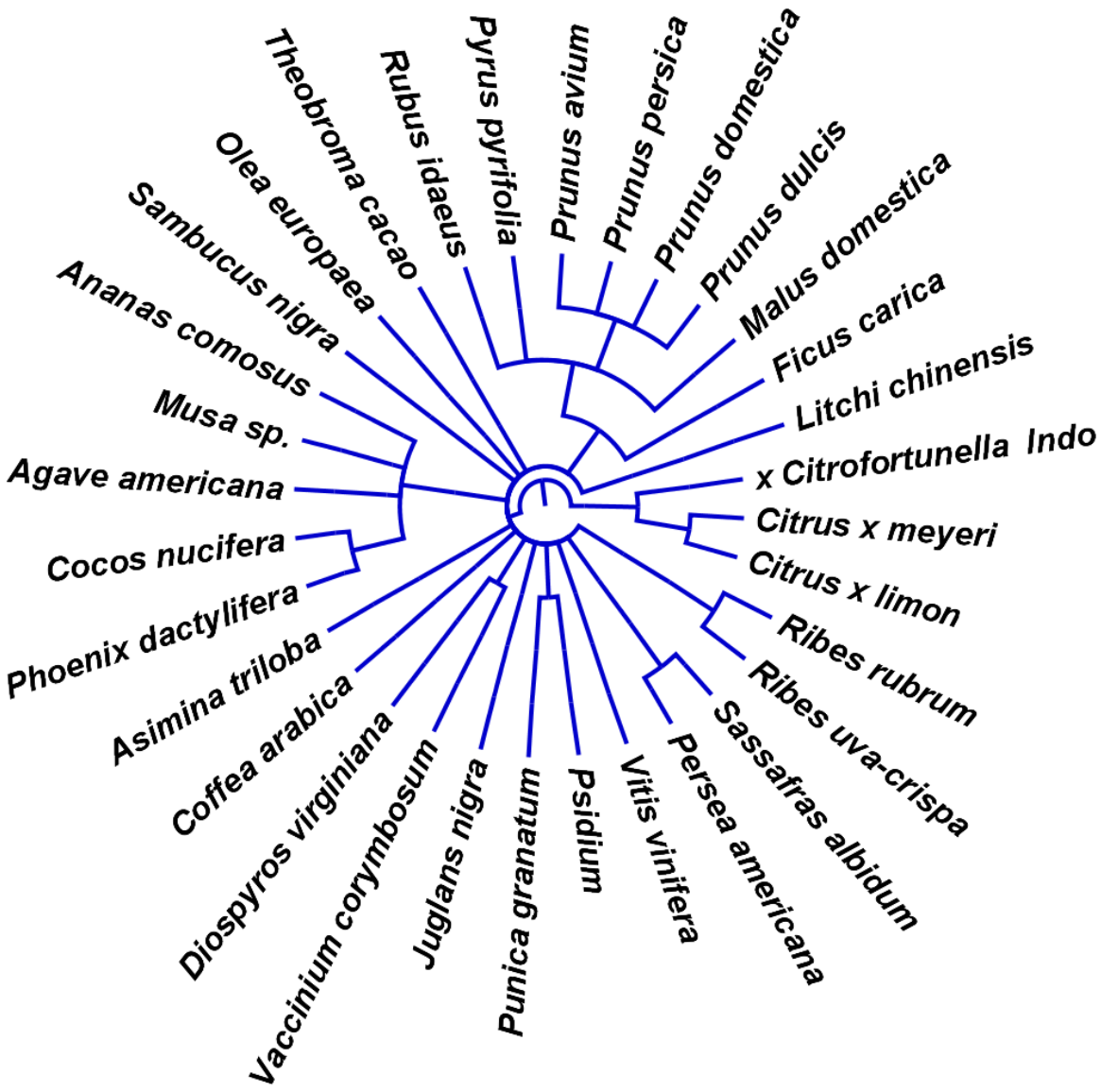

Figure 4.6. Phylogenetic tree of the 33 common horticultural crop species sampled in this study. Tree topology is based on APG III.

CHAPTER 5

Summary

Fruit removal influenced above- and belowground physiology in apple trees. In 2013, date of fruit removal did not influence glucose or starch concentrations in 1- and 2-year-old wood in December or from early spring dormancy to eight days after thinning in 2014. Crop density in 2014 also did not affect glucose or starch concentrations in 1- and 2-year-old wood at early spring dormancy or at thinning time in 2015. Fruit set was positively and linearly related to glucose concentration in 1- and 2-year-old wood for non-thinned trees, but only in 2014. In contrast to non-thinned trees, fruit set of thinned trees was not related to glucose concentration. The weak relationship observed between fruit set and glucose concentration for thinned trees in this study suggests that the level of thinning does not differ with glucose concentration at thinning time. In addition, fruit set was not related to starch concentration in 2014 or 2015. Mature apple trees may have a large capacity to store and metabolize carbohydrates to buffer against environmental stresses or experimental treatments that affect carbohydrate availability. Our results suggest that fruit set in response to chemical thinners is not directly related to early season glucose or starch concentrations in 1- and 2-year-old wood. The effects of environmental factors on underlying physiological mechanisms that result in fruitlet susceptibility to thinners and abscission should be further investigated.

Fruit removal also affected root and arbuscular mycorrhizal fungal foraging in nutrient-rich localized “hotspots”. We found that trees without fruit exhibited a more generalist foraging behavior. Root production was relatively high and non-selective in response to the type of

nutrient patch later in the season when C demand from the fruit is highest. Trees with fruit exhibited an alternative foraging behavior, and roots were considerably more selective in response to localized nutrient “hot spots”. This shift in foraging behavior resulted in about three times more root production in the inorganic-N patch than the unfertilized patch for trees with fruit. Thus, selectivity in nutrient foraging needs to be understood in the context of energy status and the relative C available. Mycorrhizal fungi were less affected by fruit removal than roots, but reduced C available to roots enhanced the ability of AM fungi to internally colonize the roots. The N source of the nutrient patch affected root proliferation differently from AM fungi, and AM extramatrical tissues were possibly enhanced by organic nutrient “hot spots”. In this study, more than 50% of the apple roots showed no evidence, internally or externally, of AM colonization. Similar colonization rates were also found in grape and peach with 56% and 64% of total root length colonized respectively (unpublished data, Lavely). Low intraradical colonization of these horticulture species may be due to soil fertility but may also indicate a low reliance on AM fungi for nutrient foraging. These results suggest that at least for N, plants rely more on root foraging than hyphal foraging to exploit nutrient-rich patches when energy reserves are limited.

Improved understanding of root responses to management practices such as fertilization, may help support greater efficiency and sustainability in perennial horticultural production systems. Separating roots into root orders or functional classes of absorptive fine roots and transport fine roots may be a useful option to characterize root responses. For example, to characterize the proportion of the root system that is primarily responsible for water and nutrient uptake in apple, 1st- through 3rd-order roots may be combined as absorptive fine roots and separated from higher-order roots. Alternatively, use of a diameter cutoff within a species, as mentioned above

with apple (0.3 mm), may effectively differentiate absorptive and transport fine roots; however, a unique diameter cutoff should be determined for each species to make functional comparisons. In conclusion, using an arbitrary diameter cutoff, such as 2 mm, to classify fine roots in the horticultural crops, apple, peach, and grape, would have led to erroneous estimates of absorptive fine roots by including both woody and non-woody roots. The differences observed in lower-order, absorptive versus higher-order, transport fine roots in the species examined highlight the potential utility of characterizing fine roots using a root-order approach in perennial horticultural crops. Moreover, perennial horticultural species vary widely in root diameter, limiting a single diameter approach for cross-species comparisons. Functional characterizations of fine roots will enhance our understanding of plant responses to management practices that affect above- and belowground interactions and whole-plant physiology. It will also provide more precise methods to evaluate the effects of practices, such as fruitlet thinning and fertilization, on belowground responses and to make broad comparisons of plant responses across planting locations and species.

Appendix

CHAPTER 3. Supporting Information

Table S1. Monthly mean soil temperature (˚C) and soil moisture content (%) in the presence or absence of apple fruit.

	Soil temperature (˚C)		Soil moisture (%)	
Monthly mean	Fruit	No fruit	Fruit	No fruit
July	24.7 (±0.4)	24.8 (±0.4)	31.6 (±3.1)	29.6 (±1.4)
August	19.2 (±1.7)	19.0 (±0.5)	30.4 (±4.0)	25.7 (±2.9)
September	13.1 (±0.4)	13.1 (±0.4)	40.9 (±0.5)	41.6 (±0.2)
October	16.2 (±1.4)	15.7 (±1.2)	37.8 (±3.5)	36.0 (±3.1)
P-value of FR	0.97		0.71	

Parenthetical values represent 1SE. The probability of significance of fruit removal (FR) is shown.

Table S2. Percentage of roots (ages 7 to 28 days old) that were uncolonized, only colonized by arbuscular mycorrhizal fungi (AMF), only colonized by non-mycorrhizal fungi (NMF), and colonized by both AMF and NMF in response to apple fruit removal and localized nutrient addition (NU; unfertilized control, inorganic N and organic N).

	Uncolonized (%)		AMF (%)		NMF (%)		AMF+NMF (%)		Uniformity of response
Treatment	Fruit	No fruit	Fruit	No fruit	Fruit	No fruit	Fruit	No fruit	*P*-value
Unfertilized, *n*=62	23 A q	24 X q	9 A p	9 X p	10 A p	11 X p	7 A p	7 X p	**<0.01**
Inorganic N, *n*=139	17 A b	29 X a	11 A bc	14 X b	12 A bc	10 X bc	6 A c	3 X c	**0.01**
Organic N, *n*=79	11 A q	44 Y p	7 A q	11 X q	13 A q	13 X q	0 B r	1 X r	**<0.01**
P-value of fruiting	0.14	**0.04**	0.50	0.40	0.80	0.70	**0.03**	0.10	

Also shown are the sample size (*n*) and *P*-values (based on χ^2 statistic) of fruit and nutrient treatment interactions of a particular colonization type (e.g., AMF, NMF). Upper case letters illustrate pair-wise comparisons within a column, and lowercase letters illustrate pair-wise comparisons within a row of a particular colonization type. Significance at $P \leq 0.05$ is in bold.

Figure S1. Root boxes were designed to measure root and fungal responses to fruit removal and nutrient addition. Root boxes dimensions were 0.7-m long, 0.7-m wide and 0.3m deep. They were installed between the two middle research trees in the four-tree experimental unit (A). Root box treatment windows faced the research trees, and windows were covered with 3-µm thick sheets of clear acetate (B). Windows were divided into two sections, and nutrient treatments were added at the acetate -soil interface. The organic N treatment (dried, ground apple leaves) at the time of application is shown (C). A diagram of research trees with fruit and root box dimensions are provided showing the N treatment windows as unfertilized control (Ck), inorganic N (IN), and organic N (ON).

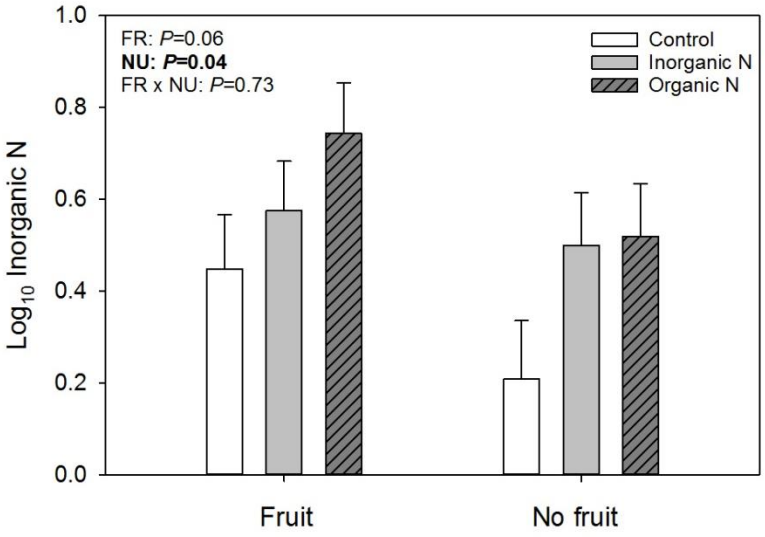

Figure S2. Total inorganic soil nitrogen (N) concentration in response to apple fruit removal (FR) and localized nutrient addition (NU). Solution was collected with micro-lysimeters in unfertilized control, inorganic-N, and organic-N soil patches, and Log_{10} transformed seasonal means are presented. Error bars represent 1SE. The probability of significance of FR, NU and the interaction is also shown. Significance at $P \leq 0.05$ is in bold.

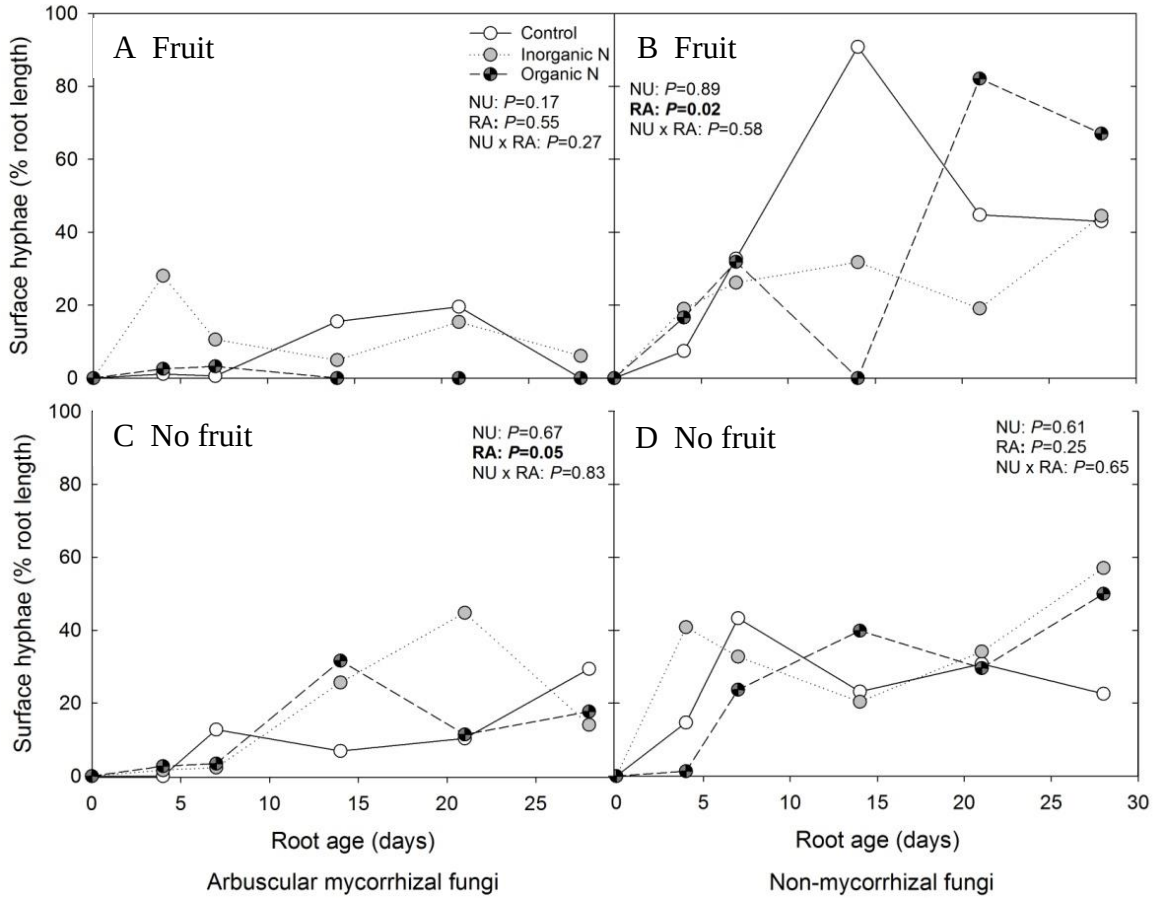

Figure S3. Mycorrhizal and non-mycorrhizal fungal hyphae on the root surface as a percentage of root length for roots of different ages (RA; newly born to 28 days old) in response to localized nutrient addition (NU) for fruit and no-fruit treatments. Surface hyphae of arbuscular mycorrhizal fungi (AMF) (A) and non-mycorrhizal fungi (NMF) (B) on roots of trees with fruit as well as surface hyphae of AMF (C) and NMF (D) on roots of no-fruit trees growing in unfertilized control, inorganic-nitrogen (N), and organic-N soil patches are presented. All percentage data was arcsin transformed for analysis. The probability of significance of NU, RA and the interaction is also shown. Significance at $P \leq 0.05$ is in bold.

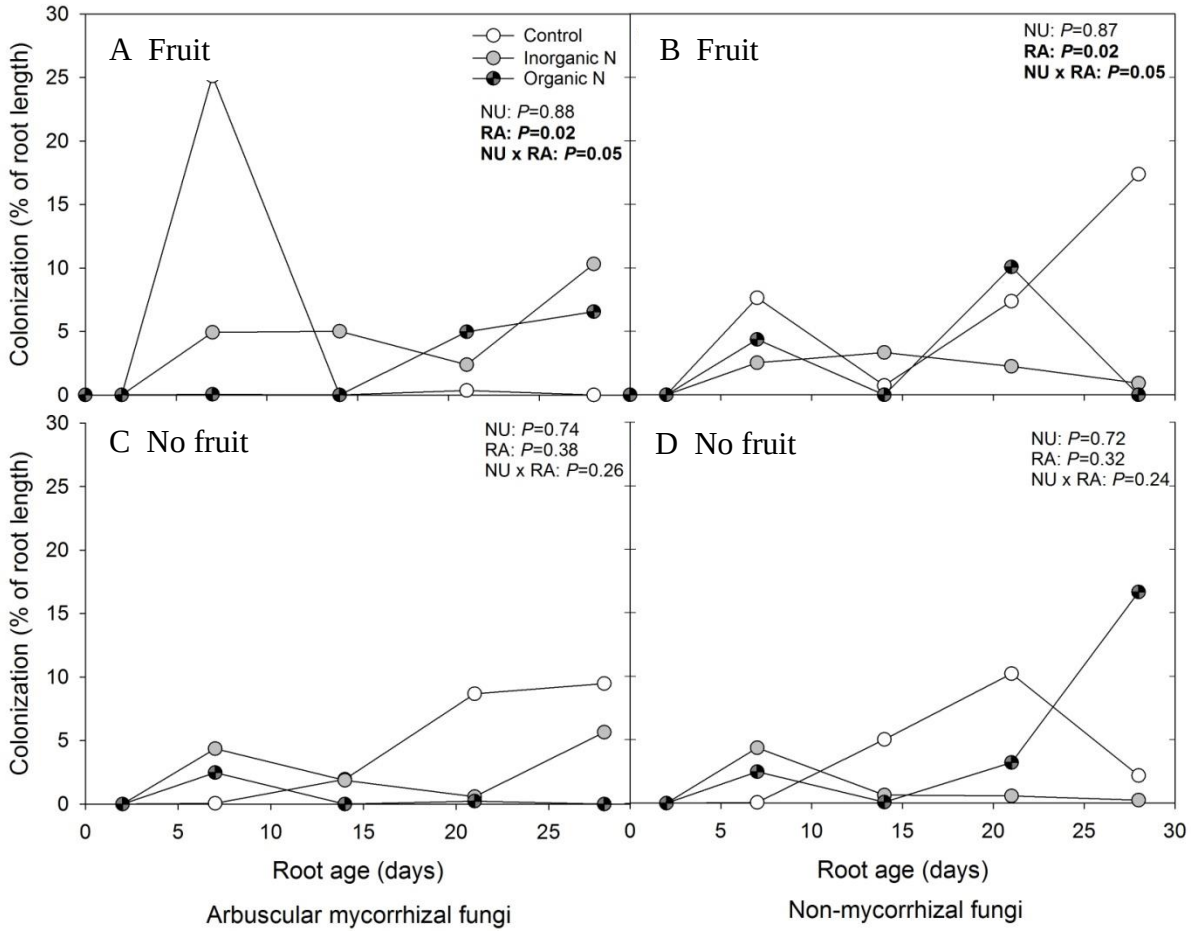

Figure S4. Mycorrhizal and non-mycorrhizal fungal colonization as a percent of root length for roots of different ages (RA; newly formed to 28 days old) in response to localized nutrient addition (NU) for fruit and no-fruit treatments. Colonization by arbuscular mycorrhizal fungi (AMF) (A) and non-mycorrhizal fungi (NMF) (B) of roots of different ages for trees with fruit as well as colonization by AMF (C) and NMF (D) of roots of different ages for no-fruit trees growing in unfertilized control, inorganic-nitrogen (N), and organic-N soil patches are presented. All percentage data was arcsin transformed for analysis. The probability of significance of NU, RA and the interaction is also shown. Significance at $P \leq 0.05$ is in bold.

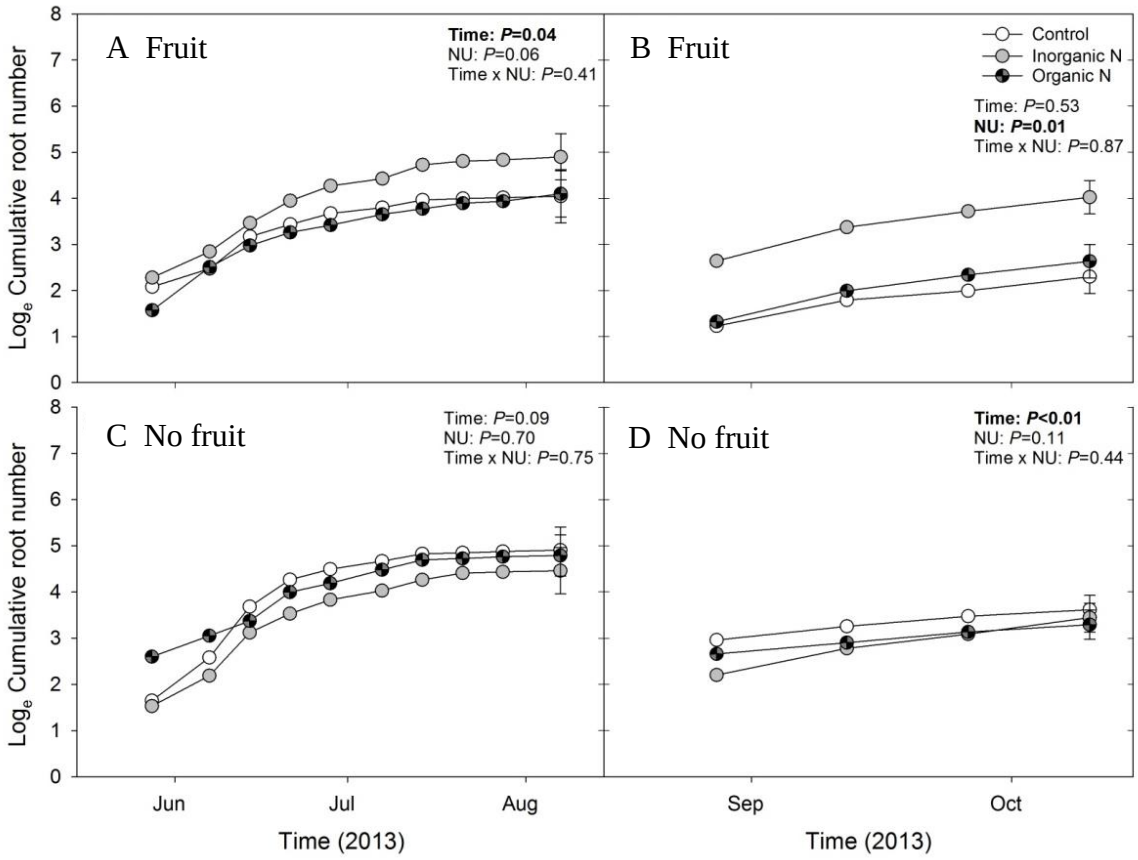

Figure S5. Cumulative root number (Log_e transformed) in response to nutrient addition (NU) over time for fruit and no-fruit treatments. Cumulative root number for trees with fruit for the first (A) and second (B) observation periods as well as for no-fruit trees for the first (C) and second (D) observation periods in unfertilized control, inorganic-nitrogen (N), and organic-N soil patches are presented. Error bars represent 1SE. The probability of significance for time, NU and the interaction is also shown. For cumulative root number, the interaction of observation period and fruit removal was not significant ($P>0.10$). Significance at $P\leq0.05$ is in bold.

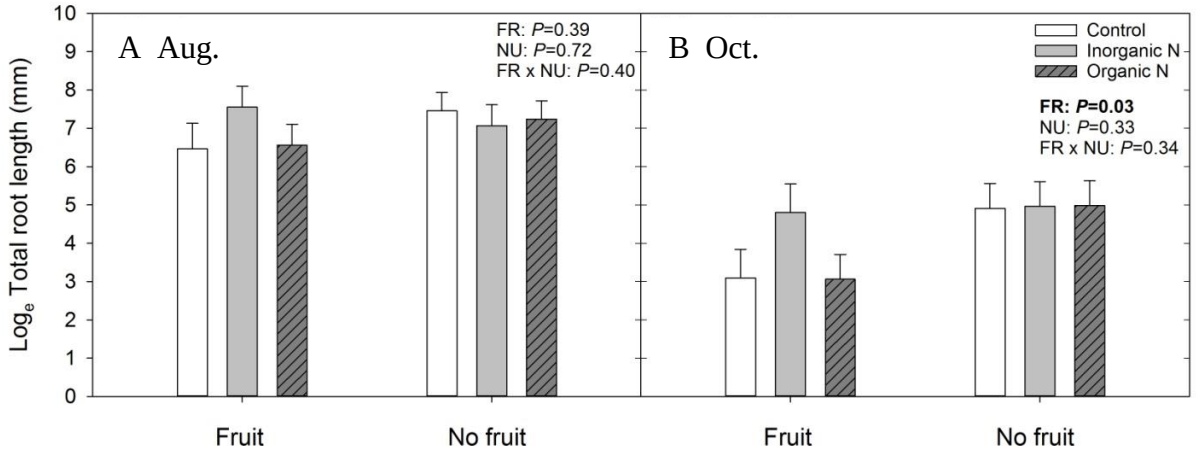

Figure S6. Total root length (Log_e transformed) in response to fruit removal (FR) and localized nutrient addition (NU). Total root length for the first (A) and second (B) observation periods in unfertilized control, inorganic-nitrogen (N), and organic-N soil patches is presented. Error bars represent 1SE. The probability of significance for FR, NU and the interaction is also shown. For total root length, observation period was significant at $P<0.01$; the interactions of observation period with fruit and nutrient addition were not significant ($P>0.25$). Significance at $P\leq0.05$ is in bold.

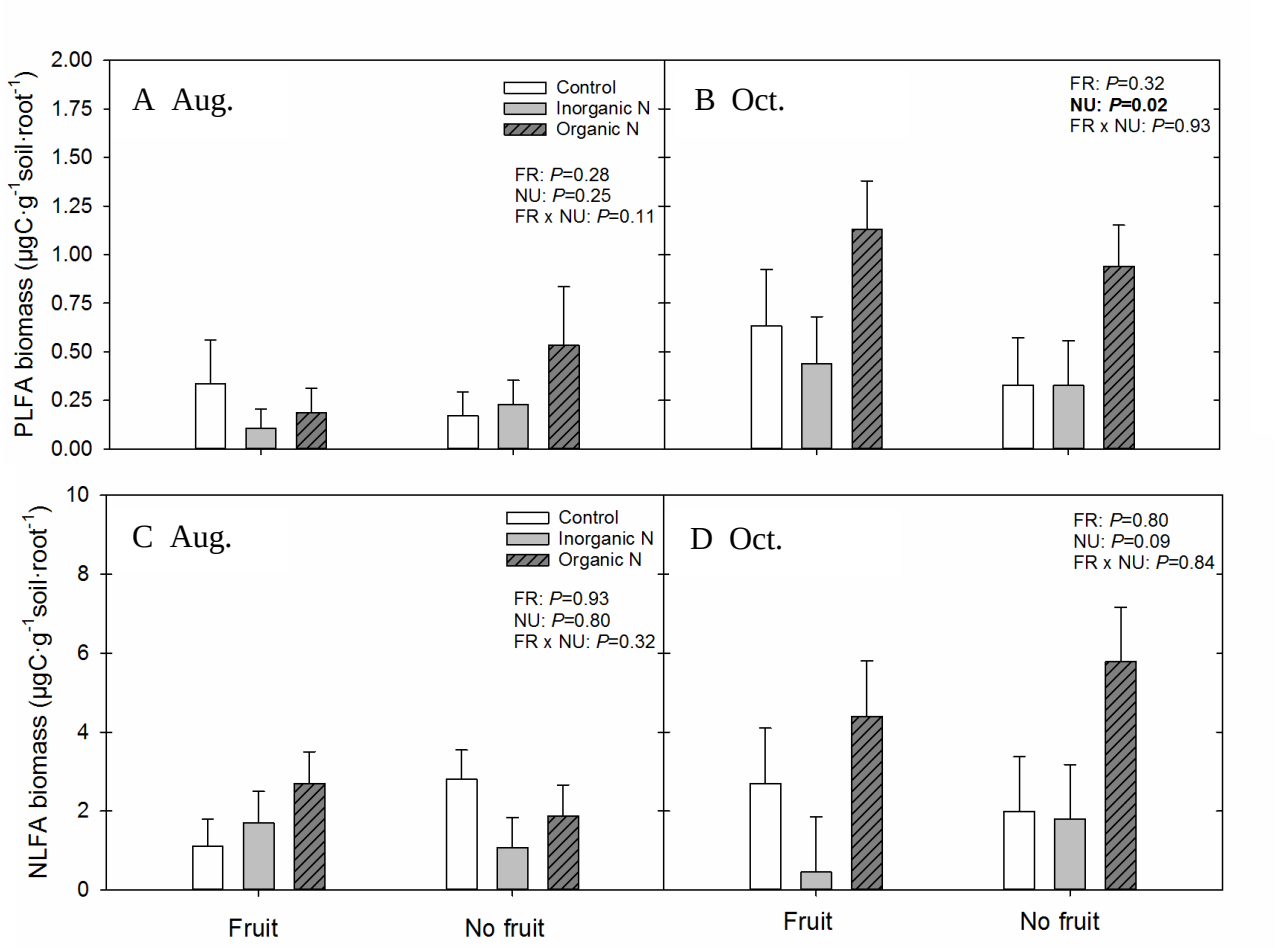

Figure S7. Root-weighted PLFA biomass (PLFA·g^{-1}soil·root^{-1} in patch) and NLFA biomass (NLFA·g^{-1}soil·root^{-1} in patch) of arbuscular mycorrhizal fungi (AMF; 16:1ω5c) within nutrient-rich patches (NU; unfertilized control, inorganic N and organic N), with or without apple fruit removal (FR) for PLFA biomass at the first (A) and second (B) observation periods and for NLFA biomass at the first (C) and second (D) observation periods. Error bars represent 1SE. The probability of significance of FR, NU and the interaction is also shown. Significance at $P \leq 0.05$ is in bold.

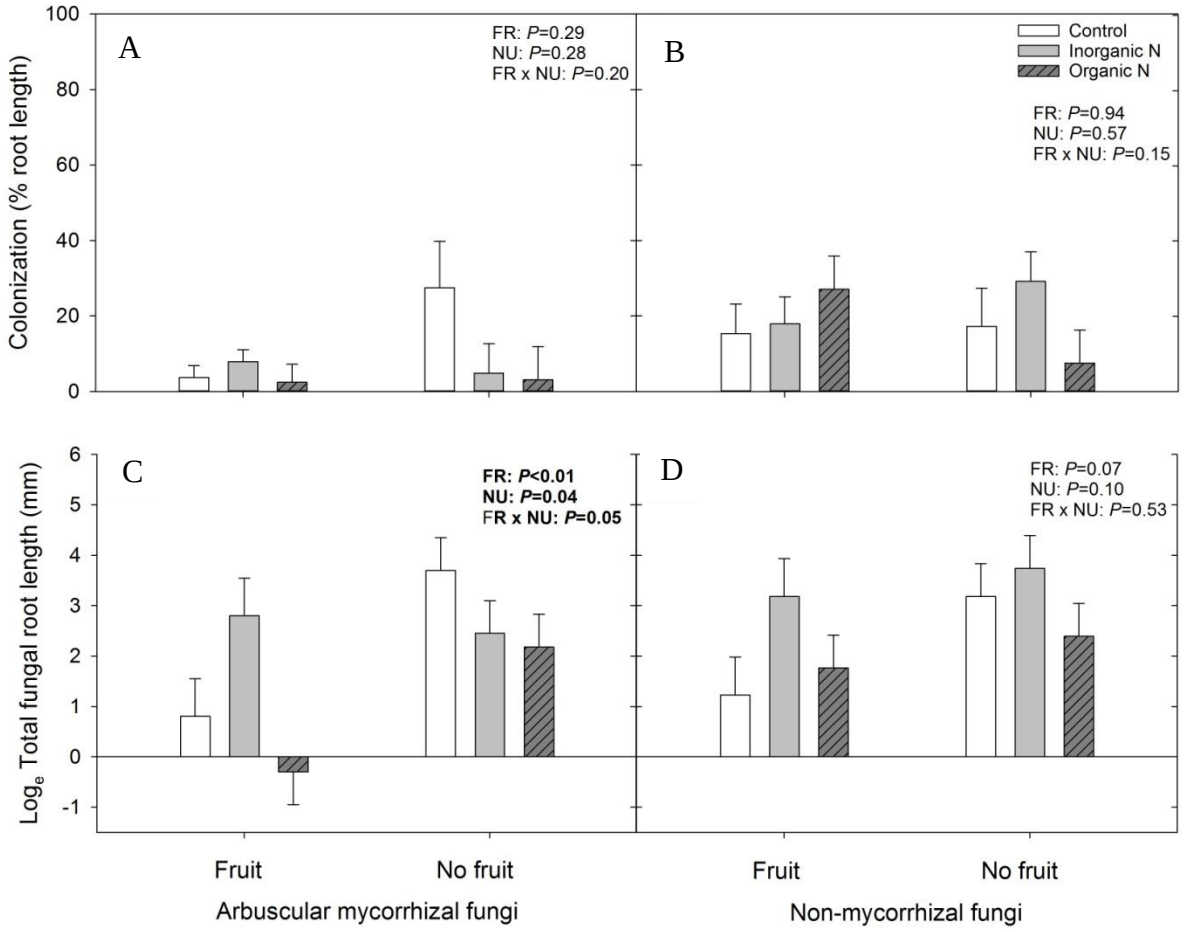

Figure S8. Mycorrhizal and non-mycorrhizal fungal colonization of roots and total fungal root length in response to fruit removal (FR) and localized nutrient addition (NU). Colonization of roots by arbuscular mycorrhizal fungi (AMF) (A) and non-mycorrhizal fungi (NMF) (B) in unfertilized control, inorganic nitrogen (N), and organic N soil patches are presented. All percentage data was arcsin transformed for analysis. Total fungal root length (Log_e transformed) is also presented for AMF (C) and NMF (D) in unfertilized control, inorganic N, and organic N soil patches. Error bars represent 1SE. The probability of significance of FR, NU and the interaction is also shown. Significance at $P \leq 0.05$ is in bold.

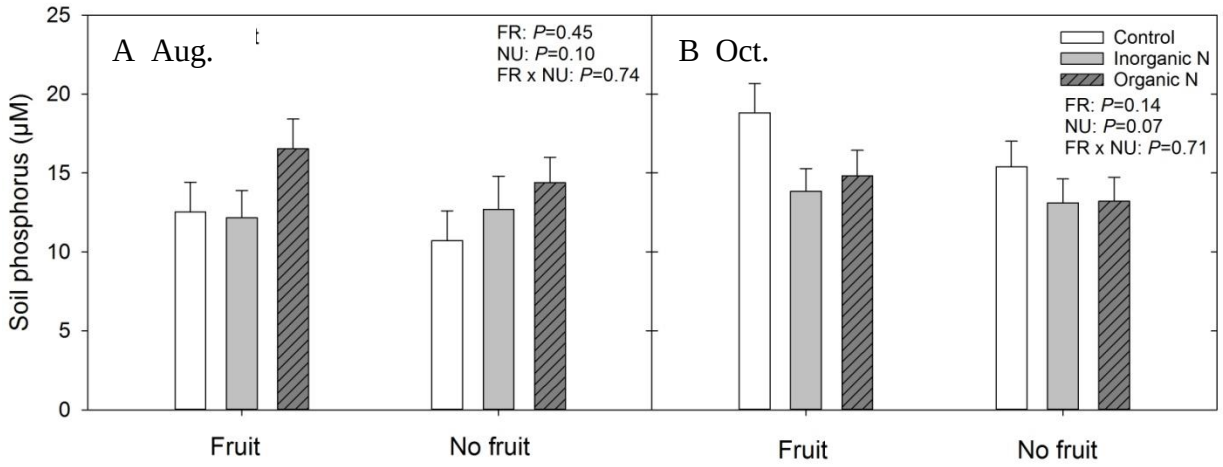

Figure S9. Soil phosphorus concentration response to apple fruit removal (FR) and localized nutrient addition (NU) in soil patches. Soil phosphorus concentration for the first (A) and second (B) observation periods in unfertilized control, inorganic nitrogen (N), and organic N nutrient-rich soil patches are presented. Error bars represent 1SE. The probability of significance of FR, NU and the interaction is also shown.

Vita

Emily K. Lavely

Education

Purdue University	Plant Genetics and Breeding	BS 2010
The Pennsylvania State University	Horticulture	MS 2013
The Pennsylvania State University	Horticulture	PhD 2013-present

Publications

Lavely EK, Zhang J, Adams TS, Bryla DR, Deforest JL, Marini RP, Crassweller R, Eissenstat DM (2018) Root and mycorrhizal fungal foraging responses to increased fruit removal in apple trees. Plant and Soil. https://doi.org/10.1007/s11104-018-3773-8.

McCormack ML, Lavely E, Ma Z (2014) Fine-root and mycorrhizal traits help explain ecosystem processes and responses to global change. New Phytol. 204(3):455-458.

Professional presentations

Lavely EK, Smith DE, Crassweller RM, Eissenstat D, Marini RP (2018) The effect of fruit removal on stored carbohydrates and the response to chemical thinning in apple. Oral presentation at the Pennsylvania State University (Abstr).

Lavely EK, Crassweller RM, Eissenstat D, Marini RP (2018) The effect of fruit removal and rootstock on stored carbohydrates and the response to chemical thinning in apple. Oral presentation at the Amer. Soc. Hort. Sci. (Abstr).

Lavely EK, Zhang J, Adams TS, Bryla DR, Deforest JL, Marini RP, Crassweller R, Eissenstat DM (2017) A Root-centric view of root-microbe interactions in apple. Oral presentation at the NC-140 Regional Rootstock Research Project, Wenatchee, WA.

Awards

NC-140 Regional rootstock research project, graduate student travel award	$1,200 (2017)
Graduate student travel award, American Society of Horticultural Science	$500, (2015)
Tag-along award, Office of international programs, Pennsylvania State University	$1,500 (2014)
Hedrick award	$300 (2013)

Student competition for best manuscript in the J. Amer. Pomol. Soc. The manuscript was published in the journal following the competition.

Academic awards

Walter Thomas Memorial Scholarship	2017
Frederick H. Brown Endowed Scholarship	2012, 2013, 2015, 2016

Professional membership

American Society of Horticultural Science
American Pomological Society