Genetic and Environmental Factors Affecting Improvement of Rootstocks for Tomato

THESIS

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By

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Abstract

Grafting is a technique that has been used for fruit trees and vine crops for thousand years. Grafting to rootstocks is becoming popular in annual vegetable production to control soil-borne diseases, replace fumigation, increase yield, increase tolerance to abiotic stress, and impart vigor. Previous research indicates that inconsistent seed quality and lack of information about rootstock-scion compatibility affect the efficiency of grafting, raises cost, and inhibits adoption of the technology. The goals of this research were to address limitations in seed quality and graft efficiency. The specific objectives were: a) evaluate genetic and environmental factors affecting quality of seed in hybrids derived from interspecific crosses, b) improve grafting success through use of adhesives, and c) determine the genetic basis of graft failure between rootstock and scion. Tomato is a model for grafting annual vegetables due to the importance of the crop and the extensive genetic resources available. To assess the potential to select for improved seed quality, experimental rootstocks were developed through pollination of cultivated (Solanum lycopersicum L.) parental lines as female parents and 11 accessions of wild species as male parents. Seed quality was evaluated based on seed size (weight) and total germinability for each hybrid produced. Maternal effects and environment determined fruit set. Specific genotype combinations and environment determined seed yield. Seed size was mainly affected by genetic components, while seed germination was affected by
both genetics and environmental factors. Seed size can be used as selection criterion in breeding program for early selection of rootstock seed quality. To improve graft success, nine different tomato rootstocks were grafted using the traditional tube method of grafting and using adhesives. Despite wide variation across rootstock genotypes and grafting environment, grafting using adhesives resulted in higher grafting success. To determine the genetic basis of graft failure, molecular markers were exploited for quantitative trait locus (QTL) analysis. An advanced backcross population (BC$_{3}$S$_{4}$), derived from two S. lycopersicum parents (H7998 × OH881199), was evaluated for graft failure, using 10 plants from each family as rootstocks with the entire experiment repeated twice. The genotyping of the BC$_{3}$S$_{4}$ population was conducted with 78 polymorphic markers for the marker-trait analysis. A second population was developed as an F$_{2}$ (H7998 × Ohio MR13) and evaluated for survival and scion height as a validation of the marker-trait associations. We identified potential regions of the genome affecting grafting failure or success on chromosome 9, and two QTL for scion height on chromosome 2 and 4. We were not able to validate these putative QTL due to the lack of overlapping markers across the two populations. The findings of this thesis work indicated that wide crosses could produce rootstocks with high seed quality. I identified key selection points in the breeding of new tomato rootstocks. At the same time, I demonstrated that control of the seed production environment is essential to obtain high quality seed from wide-cross hybrids. I also demonstrated that grafting success can be increased using adhesives in the process, and grafting failure appears to have a genetic basis, with failure associated with alleles from H7998.
Dedication

Este trabajo va dedicado:

A mis abuelitas: Q.E.P.D. Rosa Mamani de Huarachi y benedicta Martínez de Morejon

A mis padres y familia que desde la distancia siempre me apoyaron

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Publications


Fields of Study

Major Field: Horticulture and Crop Science
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Chapter 1 : Introduction

Tomato (*Solanum lycopersicum* L.) is an important vegetable consumed worldwide (Brown et al., 2005). World production of fresh tomato for 2009 was about 141 million tons planted on 4.5 million hectares in 144 countries (FAOSTAT, 2013). From 1990 and 2004 global consumption of tomatoes increased ~ 4.5% each year (Aherne et al., 2009). Fresh-tomato consumption represented about 76% of the total consumption during 1996 - 2009 (Branthome, 2010).

Soil-borne diseases and abiotic stress cause significant losses in yield every year (Rivard and Louws, 2008). Many countries have reported substantial losses of yield due to soil-borne diseases, which could be controlled by grafting to disease resistant rootstocks (Alam et al., 1994; Hasna et al., 2009; Oda, 1999). In Bangladesh, root knot nematodes caused a 53% - 62% loss of yield (Ali et al., 1994). European greenhouses have reported yield losses of up to 75% due to corky root rot (Hasna et al., 2009). As a specific example, in Sweden corky root rot causes a 30% - 40% reduction of yield in greenhouse production (Hasna et al. 2009). In Japan, soil-borne diseases can cause loss of as much as 6% of the vegetables production (Oda, 1999).
Abiotic stresses limit production of many crops. These stresses include temperature (high or low), drought, light, atmosphere, nutrients, and salinity (Haferkamp, 1987; Criddle et al., 1997; Cramer et al., 2011). Estimates of the effect of abiotic stress on global agriculture suggest that up to 70% of crop production is affected by environmental constraints (Boyer, 1982; Cramer et al., 2011). According to FAOSTAT (2006), only 3.5% of the worldwide land area is not negatively affected by any environmental factors during a cropping cycle.

The use of rootstocks offers many potential benefits, such as resistance to a wide range of pathogens in the soil, resistance to abiotic stress, and increased yield and fruit quality (Oda, 2002; Lee, 2003; Kubota et al., 2008). In the Netherlands, grafting was adopted for extending the harvest period (Lee, 2003). In the early 1990s, tomato rootstocks were not used commonly because grafting methods were inefficient and seed germination was low for interspecific hybrid rootstock (King et al., 2010). Adoption of rootstocks accelerated when interspecific crosses exhibited a high level of resistance to *Pyrenochaeta lycopersici* (corky root rot) were introduced (Lee, 1994; Oda, 1995). Later it was observed that these hybrids also had increased cold tolerance and vigor (Lee, 1994; Oda, 1995). In 1996 resistance to *Fusarium oxysporum* f. sp. *Radices-lycopersici* (crown root rot) was added to the rootstock resistance profile. Germination was improved due to commercial seed treatments, and the rootstock market grew to its current size, most of the hydroponic vegetables are grafted (King et al., 2010). Introduction of the ‘Japanese” (tube) grafting method to Europe led to large scale grafting at significantly lower cost (Oda, 1994; Lee, 1995). The use of grafting has the potential to replace the use of methyl
bromide to control disease (Miguel et al., 2004; King et al., 2010). Currently, interest in breeding for improved rootstock is increasing rapidly in the private sector due to cost effectiveness and expanded diseases resistance (King et al., 2010).

**Objectives**

The overall objective of this research was to address genetic and environmental factors limiting grafting technology using tomato as a model species. I measured the relative effect of genetic and environmental factors affecting seed production and quality when using wild relatives of the cultivated tomato for rootstock breeding. In addition, I evaluated the efficacy of adhesive in increasing grafting success between rootstock and scion. The central hypothesis of this research was that variance partitioning could be used to identify limitations in grafting technology and identify key control points for the improvement of rootstock seed, grafting technique, or genetics. The specific objectives of the research were to:

1. Evaluate genetic and environmental factors affecting quality of seed in hybrids derived from interspecific crosses.
2. Improve grafting success using adhesives.
3. Assess the genetic basis of graft failure between rootstock and scion.
Use of grafting for tomato production

The practice of grafting in annual fruits and vegetables started in the late 1920s and early 1930s as a means to control soil-borne diseases of watermelon (Oda, 2002). The gourd (*Lagenaria siceraria*) provided a compatible rootstock to control fusarium wilt (*Fusarium* spp.) in watermelon (Tateishi, 1927). In the United States, small farmers grafted vegetables onto weeds 60 years ago or more (Kubota et al., 2008). In the early 1960s, grafting was introduced into commercial production of tomato in many parts of the world (Harrison and Burgess, 1962; Bravenboer and Pet, 1962; Smith, 1966; Upstone and Finney, 1966; Smith, 1968 and Lee and Oda, 2003). In the early 1990s, grafting was re-introduced to European countries as a tool to control root diseases of soil and in hydroponic systems (Oda, 2002). More recently, use of the technique has expanded to the Middle East, North America, Central America, and other parts of the world. Currently, grafting of annual vegetables has been applied to different degrees by different agricultural sectors. For example, nearly all-hydroponic tomato production is currently from grafted plants (Lee, 1994; Kubota, 2008; King et al., 2010). In contrast, few soil-based production systems outside of Asia have adopted the technique (Kubota et al., 2008). However, interest is high and the market for grafted plants appears to be expanding in all sectors (Lee et al., 2010). Grafting is widely used in cucurbitaceous crops (cucumber, melon, and watermelon) and Solanaceous (tomato, eggplant, and peppers) (Oda, 2002).
Control of soil-borne diseases

**Chemical control**

Methyl bromide (CH$_3$Br) is one of the most widely used pesticides in the world (Shorter et al., 1995), and tomato production has the highest use, with 23% of the total used in agriculture (Braun and Supkoff, 1994; Schafer, 1999). In 1992, the parties of the Montreal Protocol listed methyl bromide as an ozone-depleting compound (Braun and Supkoff, 1994; Schafer, 1999 and USDA, 2000). In addition, this product is thought to cause health problems in exposed humans and animals (Calvert et al., 1998; Schafer, 1999; Barry et al., 2012). Recognition of negative environmental and health effects drove legislation to phase out use of methyl bromide completely by 2005 in developed countries and by 2015 in developing countries (Schafer, 1999 and USDA, 2000). Despite this legislation, methyl bromide is still widely used in many countries due its effectiveness and convenience for controlling soil-borne diseases in many crops (Akca et al., 2009; Barry et al., 2012; Walse et al., 2012).

**Non-chemical control**

Soil solarization is one alternative to methyl bromide. Solarization controls efficiently many pathogenic fungi, bacteria, weeds, and nematodes in vegetables and ornamental plants (Katan et al., 1976; Katan, 1981; Braun and Supkoff, 1994; Stapleton 2000, Tjamos et al., 2000; McSorley et al., 2009). Soil solarization involves heating the soil with sunlight or ultraviolet radiation under a transparent polyethylene cover, leading to lethally high temperatures for the pathogens (Katan et al., 1976; Katan, 1981;
Stapleton and DeVay, 1986; Stapleton, 2000; Tjamos et al., 2000; Wang and McSorley, 2008). This technique gained popularity in hot regions of Israel to control *Verticillium dahliae* and *Fusarium oxysporum f. sp. lycopersici* (Katan et al., 1976; Katan, 1981). It was later expanded to other countries, including cooler areas. For further information, see reviews (Stapleton and DeVay, 1986; Braun and Supkoff, 1994; Tjamos et al., 2000; Stapleton, 2000; Ioannou, 2001; Wang and McSorley, 2008; McSorley et al., 2009). In developing countries, solarization has become common for disinfestation of seedbeds, containerized planting media, cold frames, and tomato supports (Stapleton, 1998; Stapleton et al., 1999; Stapleton, 2000). Soil solarization is more effective in controlling soil-borne diseases and pests when combined with other methods including chemicals, organic amendments, biological control agents, and grafting (Braun and Supkoff, 1994; Gamliel and Stapleton, 1997; Stapleton, 2000; Tjamos et al., 2000; Ioannou, 2001; Bello et al., 2002). The highest use of solarization appears to be in protected crops (Cartia, 1998; Stapleton, 2000). Despite the efficiency, ease, and safety, farmers have not broadly adopted soil solarization as an alternative to methyl bromide due to its labor-intensive nature, dependency on a hot climate for effectiveness, potential interference or delay of planting, and use of plastic film must be discarded (Braun and Supkoff, 1994; Stapleton, 2000; Tjamos, 2000). This last obstacle remains an issue despite successful recycling of the plastic film (Katan and DeVay, 1991). In addition, there are some heat-tolerant pests (e.g. *Marophomina phaseolina*, *Synchitrium lagenariae* and *S. trichosanthis*), which cannot be controlled by solarization (Katan, 1981; Katan, 1987; Stapleton and DeVay, 1995; Stapleton, 1998).
**Genetic control**

Another main alternative to methyl bromide is using resistant varieties, which may be the solution for many soil-borne diseases and pests (Braun and Supkoff, 1994; Kaskavalci et al., 2009). Most contemporary hybrid tomato varies have multiple resistance to soil-borne pathogens, such as *Verticillium wilt* (race 1), *fusarium wilt* (*F. oxysporum* f. sp. *lycopersici*, races 1 and 2), and root-knot nematodes (Smith, 1944; Ioannou, 2001; Kaskavalci et al., 2009; Labrada, 2008). One of the main disadvantages of resistance breeding is that most genes are only effective against a single pathogen or one race of a pathogen (Braun and Supkoff, 1994; Besri, 2003). In addition, resistant varieties may not always be available with the complete complement of quality attributes needed for a specific market. Many landrace or indigenous cultivars lack the breeding history of contemporary varieties and therefore lack resistance. Additional research is required to create resistant and marketable varieties through conventional breeding or genetic engineering techniques. Grafting onto resistant rootstock has the potential to address concerns about chemical use while decreasing production limitations. Furthermore, this technology is considered as a component of an Integrated Pest Management (IPM), which includes many other methods of control e.g. solarization (Ioannou, 2001), bio-fumigation, intercropping (Kaskavalci et al., 2009). Grafting also avoids loss of time and loss of important traits by introducing resistance genes into modern cultivars.

Disease resistance has been a driving influence for the adoption of rootstocks for annual fruit and vegetable production. The major soil-borne diseases affecting field tomatoes around the world are bacterial wilt of tomato (*Ralstonia solanacearum*),
fusarium wilt (*Fusarium oxysporum* Schlecht. f.sp. *lycopersici* (Sacc.) Snyder & Hansen, race 1, race 2, and race 3), fusarium crown and root rot (*Fusarium oxysporum* f. sp.*radicis-lycopersici* (FORL)), verticillium wilt (*Verticillium dahliae*, races 1 and 2), *Clavibacter michiganense* (bacterial canker), Orobanche (*Orobanche ramosa*), Corky root rot (*Pyrenochaeta lycopersici*), and root knot nematodes (*Meloidogyne spp.*) (Rivero et al., 2003; Besri, 2008; King et al., 2008; Rivard and Louws, 2008; Rivard et al., 2010). Over time, grafting has been expanded to control numerous soil-borne diseases. In Japan and Korea, the objective of grafting is now to control bacterial wilt, fusarium wilt, corky root, verticillium wilt and nematode in tomato (Lee, 1994; Oda, 1999; Lee, 2003; Oda, 2007).

Various rootstocks have been created for cucurbitaceous plants. Cucurbits are commonly grafted onto an interspecific hybrid between *Cucurbita maxima* and *Cucurbita moschata* (Shintozwa) (Lee, 1994; Lee et al., 2003; Oda, 2007). Other cucurbitaceous rootstocks (Watermelon, Cucumber and Melon) have been reviewed (Oda, 1999; Lee, 2003; Oda, 2007; Lee et al., 2010). Grafting in annual crops was initiated with cucurbit annual vegetables and by now, these crops are the most grafted vegetables, especially in countries where the land use is intensive.

In Solanaceous vegetables, *S. Lycopersicum* or interspecific hybrids (*S. lycopersicum × S. habrochaites*) are widely used as rootstock for tomato. Early reports from India demonstrated that ‘CRA 66’, a wild eggplant rootstock, could reduce bacterial wilt incidence in tomatoes, increasing survival rates from 0% (control) to 100% of the grafted plants at last harvest time (Tikoo, 1979). For more details about other solanaceous
vegetables (Eggplant and Sweet pepper) see previous reviews (Yamakawa, 1982; Lee, 1994; Oda, 1999; Lee et al., 2003; Oda, 2007 and Lee et al., 2010). Corky root was controlled using ‘Beaufort’ tomato rootstock, where the grafted plants were healthier and more uniform compared to non-grafted plants (Hasna et al., 2009). Southern blight (Sclerotium rolfsii) and root-knot nematode (Meloidogyne spp.) can also be minimized by grafting (Rivard et al., 2010). The interspecific hybrid rootstock ‘Multifort’ (De Ruiters Seeds, Bergschenhoek, The Netherlands; Now Monsanto, St. Luis, Mo.) shows tolerance under severe infestation of root-not nematode and the hybrid rootstock ‘Survivor’ (Takii Seeds, Salinas, CA) significantly reduced root galling compared to ‘Multifort’ (Barrett et al., 2012). Given the effectiveness of rootstocks to control disease of the root system, it is not surprising that grafting is used commercially to control soil-borne diseases under different environments (Di Gioia et al., 2010; Rivard et al., 2010 and Louws et al., 2010).

**Rootstock tolerance to abiotic stress**

Cultivated tomato lacks variation and specific adaptation to low temperatures (Paul et al., 1984; Nieuwhof et al., 1999; Schwarz et al., 2010). Temperature is an important environmental factor that affects tomato production (Hansen et al., 1994; Venema et al., 2008; Schwarz et al., 2010; Venema et al., 2010). For soil temperature, the critical root temperature for tomato range from 15 °C to 34 °C (Martin and Wilcox, 1963; Zijlstra and DenNijjs, 1987). Low root temperature affects the productivity of the plant by (1) delaying initiation of growth, (2) restricting water movement to roots, (3) decreasing permeability of the membrane on the root surface, and (4) delaying opening of the
stomata with a resulting reduction of daily photosynthesis (Haferkamp, 1987; Bloom et al., 2004). Wild species offer potential sources to increase the genetic variation and phenotypic variation for low temperature tolerance in cultivated tomato (Venema et al., 2008; Bloom et al., 2004). For example, *Solanum habrochaites* grows in the Andes (from 500 up to 3300 meters above sea level) and grows at chilling temperatures that are detrimental for cultivated tomatoes (Vallejos and Tanksley, 1983; Bloom et al., 2004; Venema et al., 2008). The cold tolerant accession LA 1777 (*S. habrochaites*) was used as a rootstock under two temperatures 25 °C and 15 °C (Venema et al., 2008). This study demonstrated that it is possible to graft a cultivated tomato onto a wild species without creating hybrids. However, the study did not evaluate the yield or other characteristics of the grafted plants. The only parameter evaluated was root development under low temperature, and grafted plants onto *S. habrochaites* LA 1777 had better developed roots than the self-grafted plants (Venema et al., 2008). Thus, grafting could combine a marketable tomato with rootstocks tolerant to low or high temperatures, thereby stimulating better growth and development under either thermal or chilling conditions.

High temperatures (greater than 35 °C) can reduce fruit set in tomato; physiologically it inactivates the metabolism of the plant and causes a reduction of foliar-biomass (Haferkamp, 1987; Hansen et al., 1994; Rivero et al., 2003; Schwarz et al., 2010). For grafting, the use of more distantly related species as a source of new variation becomes possible. For example, wild eggplant rootstock for tomatoes has allowed plants to cope with hot-wet seasons, including flooding, waterlogging and high temperature (Black et al., 2003; King et al., 2010). Rivero et al., (2003) grafted tomato onto a heat
tolerant rootstock (*L. esculentum* cv. ‘RX-335’), and demonstrated g resistance to high temperatures which resulted in superior plant biomass in grafted plants compared to non-grafted plants.

The soils of many production regions around the world are affected by high concentrations of salt (Yamaguchi and Blumwald, 2005; Munns, 2002; Martinez-Rodriguez et al., 2008; FAOSTAT, 2009; He et al., 2009; Estañ et al. 2005; Colla et al., 2010; Venema et al., 2010). Salinization is often caused by irrigation with poor-quality of water (Estañ et al., 2005; Santa-Cruz et al., 2002; Fernandez-Garcia et al., 2004; Venema et al., 2008). Because salinity affects many physiological and biochemical aspect of plants including ion balance, ion toxicity, and hyperosmotic stress (Santa Cruz et al., 2002; Cuartero et al., 2006; Colla et al., 2010; Fernandez-Garcia et al., 2004), lands with high salt concentrations are no longer useful for agriculture. As with heat and cold resistance, wild species offer a potential source of tolerance to salt stress (e.g. *L. pennellii* accession LA0716). A genetic study based on cultivated tomato and a wild species (LA0716) populations estimated the proportion of genetic to environmental variance (heritability) for salt tolerance to be in the range of 0.3 - 0.45, suggesting that salt tolerance can be improved through genetic selection (Saranga et al., 1992). Wild tomatoes have salt tolerance genes, but it may be difficult to move this trait due to the complexity of the genetic architecture, which appears to be a quantitative multigenic-controlled trait (Jones, 1985; Saranga et al., 1992; Santa-Cruz et al., 2002; Estañ et al., 2005). Rootstocks offer a potential solution to reduce the negative effects of high salt concentration and avoid damage by salinity (Cuartero et al., 2006; Martinez-Rodrigues et
Santa-Cruz et al. (2002) suggested that grafting might be a valid technique for tomato under saline conditions. Estañ et al. (2005) and Martinez-Rodriguez et al. (2008) demonstrated that grafting onto appropriate rootstock could reduce ionic stress. Grafting provides an alternative way to confer salt tolerance on the scion through the rootstock.

The high chemical inputs used in contemporary agriculture (such as fertilizers, herbicides, and other chemicals), has resulted in high concentrations of heavy metals, pesticides, and nitrates in the soil (Savvas et al., 2010). Depending on the rootstock genotype, grafted plants may grow under conditions of heavy metal contamination by minimizing transport of heavy metals (Cu, B, Cr, Cd) into the fruit or leaves, using a mechanism of restricted uptake (Estañ et al., 2005; Arao et al., 2008; Savvas et al., 2009; Savvas et al., 2010).

This review of the existing literature suggests that grafting has many benefits, from increase yield and fruit quality, disease resistance, abiotic stress tolerance, and ion and nutrient uptake efficiency. Rootstocks can improve resistance to diseases of the root system and they can impart tolerance to abiotic stress. Rootstocks can also restrict or enhance the uptake of specific nutrients compared to non-grafted plants, with some combinations having higher ability to take up nutrients and others restricting specific transport (Leonardi and Giuffrida, 2006). Given the diversity of potential uses, there is a need to design knowledge-based strategies of breeding rootstocks with high seed quality, and increase grafting success using new methods of grafting. In addition, it is important to understand the genetic basis of rootstock-scion grafting failure.
Goals of breeding tomato

Over time, the goals of tomato breeding have changed (Grandillo et al., 1999). In the 1970s, the most important trait to breed for was increased yield. In the 1980s, extending tomato fruit shelf life was a priority. At present, a goal for many tomato breeding programs is sensorial and nutritional quality (Grandillo et al., 1999; Bai and Lindhout, 2007). Quality is a general term must be applied to specific markets, and includes physical properties that contribute to flavor and compounds that provide nutrition and health benefits (Rouphael et al., 2010). With shifting breeding goals, it is sometimes difficult to combine traits. Thus, grafting offers an opportunity to combine resistance breeding with multiple quality traits.

Studies in many locations and countries have reported that yield was increased for a variety of rootstock-scion combinations. These increases are reported as an increased number of fruits per plant and fruit weight (Turhan et al., 2011). For example, the variety ‘Lemance’ grafted onto ‘Beaufort’ rootstock showed higher yield compared to non-grafted plants (Poganyi et al., 2005). Plants grafted onto two different rootstocks (‘He-man’ and ‘Primavera’) produced more fruit; increasing yield up to 32.5% (greenhouse) and 11.1% (field) (Khah et al., 2006). When ‘Cecilia F1’ was grafted onto three rootstocks (‘Beaufort’, ‘He-man’ and local Syria tomato); productivity increased up to 21% (Mohammed et al., 2009). The combination ‘Beaufort’ used as the rootstock and ‘Monroe’ as the scion showed an increase in yield from 3.37 kg.plant\(^{-1}\) non-grafted to 4.46 kg.plant\(^{-1}\) grafted plants (Kacjan-Marsic and Osvald, 2004). Yield increases close to 20% compared to non-grafted plants have been reported (Gebologlu et al., 2011).
Sweden, plants grafted onto ‘Beaufort’ rootstock showed yield increases and more uniform fruit size (Hasna et al., 2009). Gebologlu et al., (2011) showed data that yield increased from 13.85% to 37.72%, compared to non-grafted plants. However, reported yield increases are not universal. For example, ‘Belle’ as a scion grafted onto ‘PG 3’ rootstock had lower yield (Kacjan-Marsic and Osvald, 2004). In our work with 36 rootstocks grafted to Celebrity and grown in three, soil-based, open field production trial, grafted plants had an increase in yield relative to checks. However, only ‘Beaufort’, ‘Maxifort’, and ‘VFNT cherry’ of these were statistically significant, and several had significantly lower yields relative to non-grafted or self-grafted controls. Delayed maturity has also been observed on grafted plants.

Multiple reports have shown effects of grafting on fruit quality; some researchers have found positive effect, other negative effects of grafting on fruit quality. For example, when “Beaufort” was used as rootstock and ‘Lemance’ as a scion, soluble solids °Brix, carbohydrates, and organic acids were lower in grafted plants than those from non-grafted ones (Pogonyi et al., 2005). On the other hand, the rootstock cv. ‘Radja’ increased fruit quality (soluble solid content and titratable acid) of fruit from scions (Flores et al., 2010). A hybrid rootstock based on Solanum cheesmaniae × cultivated tomato improved the soluble solids content and titratable acid (Flores et al., 2010). The increase in total dissolved solids and decrease in lycopene content was reported (Mohammed et al., 2009). Turhan et al. (2011) reported no effects of grafting on lycopene and pH content. In a study by Gebologlu et al. (2011), vitamin C, water-soluble dry matter, and titratable acidity were not significantly affected by different rootstock
used. Moreover, other studies have failed to observe a change in lycopene content on grafted plants under field or greenhouse conditions (Rouphael et al., 2010). Some studies reported negative effects of grafting. A reduction in vitamin C and phenolic content was reported in grafted plants (Vrcek et al., 2011). Similar to yield, studies indicate a range of responses from improved quality to negative effects. Thus, establishing which combinations of rootstock, scion, and environment improve desired traits should be a key focus of investigation.

**Hybrid seeds**

The use of hybrid seeds has increased from the late 1960’s to the current situation where hybrids dominate markets due to advantages compared to open pollinated varieties. Hybrid cultivars have vigor, uniformity, diseases resistance, stress tolerance, and desirable horticultural traits (Shull, 1911; Maxon-Smith, 1966; Rick, 1980; Opeña et al., 2001). Charles Darwin (1876) first observed hybrid vigor or heterosis in many plant species including tomato. Later, Shull defined the concept of heterosis as superior performance of a hybrid genotype in maize resulting from the cross of two inbred lines (Shull, 1908; Shull, 1911).

In tomato, studies on heterosis were initiated almost at the same time as in maize (Hedrick and Booth, 1907; East and Hayes, 1912). Heterosis has not been a driving force in tomato breeding. Rather, hybrids were used by the seed industry to protect germplasm from misappropriation. Hedrick and Booth (1907) conducted a study of hybrid vigor on tomato by performing crosses of different varieties and growing the
progeny next to both parents. They observed that the F1 progeny had higher performance in terms of growth, leaf color, and fruit production. Although heterosis in tomato is widely documented in the academic literature, with many studies having demonstrated high levels of heterosis for yield and yield component (Kravchenko, 1990; Suresh et al., 1995; Atanassova and Georgiew, 2007), there are few documented examples of heterosis for economic traits using commercially relevant germplasm. The disconnect between observed heterosis in growth and marketable yield is not an issue in rootstock breeding as heterosis in growth of the root system can be exploited, despite the fact that most rootstocks do not produce marketable fruit. Studies also demonstrated that heterotic performance might enhance the ability of plants to cope with an unfavorable environment (Khanna-Chopra et al., 1993), and increases fruit quality (Patil and Patil, 1988) and uniformity (Yordanov, 1983; Atanassova and Georgiew, 2007). Thus, the use of hybrid seeds in grafting technology has been expanded due to its high performance in different environments.

**Seed quality**

The definition of seed quality consists of several components, including, analytical, genetic or species purity, cultivar purity, germination capacity, vigor, size, uniformity, moisture content, and freedom from weeds and diseases (Desai, 2004). High-seed quality represents high profits for farmers and growers. Therefore, seed producers have responsibility to produce high seed quality.
As commonly described in tomato, the use of seed directly from wild species or from hybrid seed derived from crosses to wild species may negatively affect seed quality. In tomato, the standards of high quality seed are genetic purity of 99%, germination rate above 85%, and moisture content less than 8% (International Seed Federation, 2013). Wild species and hybrids (wild × cultivated tomato) were evaluated for uniform germination, and the variation was large (Ibrahim et al., 2001). The germination percentage of wild Solanum species and hybrids ranged from low rates up to 86%. Large variation in germination exists between wild species and hybrids (wild × cultivated tomato), ranging from 8% to 86% (Ibrahim et al., 2001). These observations are not surprising as part of the domestication process involves selection for uniform germination.

The use of F₁ hybrids as tomato rootstock has become common in tomato grafting. Early rootstock hybrids of tomato were produced using L. esculentum as a female and L. hirsutum var ‘Glabratun’ as a male (currently S. lycopersicum and S. habrochaites, respectively) (Bravenboer and Pet, 1962). In 1966, ‘GCR 66’ was issued as a new parent for rootstocks (Maxon-Smith, 1966). This new parent came from the combination of two lines with specific traits (Green stem and potato leaf positional sterile). Rootstocks derived from ‘GCR 66’ contained resistance to verticillium wilt (Ve) and fusarium wilt, race 1, which comes from the donor parent S. habrochaites (Maxon-Smith, 1968).
Reproductive isolation barriers

Hybrids from wild species and cultivated species increase the ability to combine disease resistance, but may reduce seed quality. Wild species have poor seed germination rate, poor uniformity in seed germination and strong seed dormancy (Ibrahim et al. 2001). Gibberellic acid (GA3) can be used to break the dormancy and encourage uniform seed germination; however, this technique is difficult to apply at the grower level.

Wide crosses offers the possibility of combining specific traits such as resistance to biotic and tolerance to abiotic stress. At the same time, seed quality may suffer decay due to reproductive barriers. Both genetic and ecological barriers have a role. Ecological isolation, temporal and seasonal isolation, mechanical isolation, may prevent fertilization in nature (Stebbins, 1958; Sano and Kita, 1978; Hadley and Openshaw, 1980). Genetic barriers may result in hybrid sterility, when two species may succeed in producing a viable even vigorous F1, but the structural differences between parental chromosomes causes hybrid sterilization (Hadley and Openshaw, 1980; Rieseberg, 1997). Although wide crosses offer potential advantages of hybrid vigor, genetic barriers may also lead to poor performance of hybrids resulting from such crosses. Thus, selection based on seed quality must be investigated to create new hybrids using wild relatives as a germplasm variation.

Grafting methods

Many grafting methods have been developed over time and specialized for each crop (Oda, 1999). In tomato, “cleft grafting” has high success, as defined by the number
of plants that survive the grafting process (Oda, 1995), but this method is labor intensive. In the late 1990s, a new grafting method was introduced in Japan, called “tube grafting” or the “Japanese method” (Oda, 1995). Using this new method it is possible to graft younger plants at a rate of 300-500 grafts per hour, depending on the worker’s skill (Kubota et al., 2008). The advantage of tube grafting is that it is faster than cleft grafting because it requires only one cut, instead of three cuts (Oda, 1994; Oda, 1995; Oda, 1999). The tube grafting method also frees space in the healing chamber because smaller plants may be grafted. The use of tube grafting has allowed for the creation of robotic systems, which accelerate the grafting processes and have success rates of over 93%, (Oda, 1995; Chen et al., 2010). Despite of its advantages of speed and space, this method is restricted to similar stem size of rootstock and scion. Sometimes it is difficult to obtain uniform seedlings without using an extra seeds.

In addition, adhesives such cyanoacrylate have been used in grafting. Oda and Nakajima (1992) reported the used of adhesives to graft Chinese cabbage onto turnip. Adhesives have been used to graft cucumber, eggplant, and grape (Oda, 1995). The use of cyanoacrylates in agriculture parallel those in medical applications to close wounds (Quintino and Pires 2004; Bozkurt and Saydam, 2008), in veterinary medicine (Andrade et al., 2001; Albuquerque et al., 2006; Endo et al., 2007), in dentistry (Grisdale, 1998; Leggat et al., 2004), and in aesthetic facial plastic surgery (Kamer et al., 1989). Using cyanoacrylates to join biological tissues is both inexpensive and easy. Using adhesives could help to improve efficiency and may be utilized in the automation of the grafting process using robotic systems.
**Grafting failure**

Many woody ornamentals, most fruit trees, and recently annual vegetables are propagated by grafting. Sometimes the graft union fails, resulting in the main stem breaking off, dieback, poor growth or death of the grafted plant (Hartmann et al., 1990; Andrews and Marquez, 1993).

The success or failure of grafting depends on various factors including taxonomy, environment, availability of oxygen and water, physiological stage of rootstock/scion, herbicide toxicity, the skill of the grafter, mechanical damage of the graft union, and graft incompatibility (Andrews and Marquez, 1993). Graft failure occurs when the rootstock and scion are partially or completely separate from each other. However, it is often difficult to establish the exact cause of the graft failure. Grafting failure often occurs at early stages (Martinez-Ballesta, 2010), which can be classified into three categories: (1) failed bud growth, (2) mechanical obstruction at the union, and (3) abnormal union structure (Herrero, 1951; Andrews and Marques, 1993). Grafting failure may also occur in later stages such as the fruiting stage.

In plants, the more closely related rootstock-scion are (taxonomy), the better the chances for the graft to be successful (Copes and Oliver, 1970). A successful graft begins a sequence of events during the healing process including: (1) callus proliferation from rootstock and scion, (2) callus bridge formation (3) vascular differentiation, and (4) production of secondary xylem and phloem (McCully, 1983; Moore, 1984; Andrew and Marquez, 1993; Kawaguchi et al., 2008). However, grafting can fail at healing process or when plants are fully-establish with limited warning. When tomato cultivars and wild (S.
Habrochaites species were used as a rootstock in eggplant grafting, the grafting success was low (Gisbert et al., 2011), this might be attributed to taxonomic incompatibility between graft partners or other reasons.

Grafting incompatibility is often misused as a synonym of “grafting failure” or vice versa. Many definitions of graft incompatibility are based on physiological and biochemical processes (Andrew and Marquez, 1993) or physiological intolerance at the cellular level (Moore and Walker, 1981). Often grafting incompatibility is used to mean failure or interruption of the development of the new grafted seedling. This interruption can occur for number or reasons, including taxonomic differences, cellular recognition and diseases present in the grafted partners (Garner, 1979; Andrew and Marquez, 1993). However, in the literature both terms are used as a synonym, thus, define that grafting failure can be caused by grafting incompatibility, and this former has specific reasons.

The normal growth of a grafted plant may be interrupted at any stage of development due to incompatibility between scion and rootstock. Incompatibility could be directly related to undergrowth or overgrowth of the scion relative to the rootstock (Lee, 2007). The rootstock is the portion of the plant that controls the uptake, synthesis, and translocation of water and minerals from the soil and the scion must be able to transport and use what the rootstock delivers (Lee and Oda, 2003). The perfect combination of these parts results in a successful plant that can respond to both abiotic and biotic stress in a given environment without decreasing yield or fruit quality.

In grafting tomatoes, one of the main limitations is thought to be Tobacco Mosaic Virus (TMV) infection. There are three different resistant alleles to TMV: Tm-1,
Tm-2 and Tm-2a. Tm-1 is symptomless or tolerant, Tm-2a confers a hypersensitive response, and Tm-2 is intermediate (Hall, 1980; Yamakawa, 1982; Oda, 1995; King et al., 2010). In 1981, Yamakawa suggested different combinations of these alleles for tomato grafting to avoid problematic combinations. When the rootstock and the scion have the Tm-1 allele, the probability of graft to success is higher. The same is true with Tm-2 and Tm-2a; both the scion and rootstock must have the same allele. Problems could occur when the rootstock has Tm-1 and the scion Tm-2 or vice versa. The most problematic combination could be when the rootstock has Tm-2a and scion has Tm-1. In this case, the virus may be transmitted from the rootstock to the scion inducing a systemic necrosis. In addition, the graft may be lost if the rootstock has Tm-1 and scion has Tm-2a (Yamakawa, 1982; Oda, 1995; Morra, 2004; King et al., 2010). Therefore, it is important to understand the different mechanisms that may affect graft failure.

Alternatively, grafting compatibility is the ability of two plants (scion and rootstock) to grow successfully and reproduce as a single plant after they are joined (Hartmann et al., 1990; Santamour and Frank, 1988). Marketable rootstocks must be compatible with scions without causing negative effects on yield or fruit quality. Many of the tomato rootstocks available in the market originated from pre-existing germplasm; few were developed by making crosses to generate variation and selecting superior genotypes, especially using multiple *S. habrochaites* wild lines (King et al., 2010). The factors that determine how a rootstock and scion interact to produce a strong graft are still not well understood. Most of the studies focused mainly on the physiological and biochemical factors of the graft formation, especially in tree species (Moore, 1983;
Andrew and Marquez, 1993; Kawaguchi et al., 2008). For that reason, it is important to investigate the genetic basis that causes graft failure, using tomato as a model plant for annual grafted crops.

Grafting quality and efficiency depend upon many factors, but the rootstock uniformity is a major contributor. The uniformity of the rootstock depends upon seed germination, which is affected by seed quality. In addition, grafting failure decreases the efficiency of the technology, and it depends on many factors, from the environment to the genetic level of failure. Therefore, it is important to answer the following questions (1) Can genetic selection improve rootstock seed quality? (2) Can the environment of seed maturation improve rootstock seed quality? (3) Can the use of adhesives increase grafting success? finally (4) Do rootstock and scion failure have a genetic basis? The following research will aim to answer these questions.

**QTL-analysis**

In the last decades, genomic technology advanced in gigantic steps on creating techniques and strategies, to understand the molecular level of the plant function (Somerville and Somerville, 1999; Hamilton and Robin Buell, 2012). A Quantitative Trait Locus (QTL) is a region of the genome containing an allelic difference that causes a change in phenotype. Many agriculturally important traits exhibit complex genetic architecture, including phenotypes including seed yield, plant architecture and many abiotic tolerances (Burr et al., 1983; Lamkey and Lee, 1993).
Molecular markers are signs along the organism DNA that indicates genetic traits or genetic differences i.e. polymorphisms, which distinguish alleles in a population. Multiple types of markers have been created from hybridization based, polymerase chain reaction, sequencing and DNA array (Agarwal et al., 2008; McCouch et al., 2002; Rafalski, 2002; Staub et al., 1996; Phillips and Vasil, 2001). Microsatellite or simple sequence repeat (SSR), insertion/deletions (INDELs) and single nucleotide polymorphism (SNP) are sequence-based DNA markers. These makers have been widely used in crop breeding, in creation of molecular maps (genetic and physical maps) and other genetic studies and applications (Litt and Luty, 1989; Weber and May, 1989; Varshney et al., 2007; Phillips and Vasil, 2001, Robbins et al., 2009). SNPs are the most common type of sequence variation in plant species (Lipshutz et al., 1999; Rafalski, 2002; Baird et al., 2008), in tomato this type of markers has been extensively applied in many studies and SNP arrays have created as well (Sim et al., 2009; Robbins et al., 2011).

QTLs with large effects on phenotypic variation are the easiest to identify and analyze. However, not all QTL have large effects, furthermore, most QTL have small average effects on complex traits (Mackay, 2001). Thus, while theory and experiment suggest that a large fraction of the variation of many phenotypes will be explained by QTL with smaller effect sizes (Fisher, 1930; Paterson, 2010; Shao et al., 2008; Yang et al. 2011), our current understanding of complex traits is based primarily on analyses of QTL with the largest effect sizes.

There are many factors affecting the efficiency of QTL mapping including (1) number of genes involved in the trait under control, (2) heritability of the trait (higher
better) (3) type and size of mapping population (4) density and coverage of markers, and (4) statistical methodology applied (Lippman et al., 2007). The structure of population determine their effectiveness for decipher the genetic component of a trait and their application in plant breeding. Traditional population structures for genetic studies require parents that differ widely in the trait with the effectiveness of a population determined by the separation of parents (segregation) (Fisher, 1919). There are multiple methods for QTL mapping studies to detect QTL. Single Marker Approach, is often referred as the single factor analysis of variance, and has been used widely, mainly with isozymes (Tanksley et al., 1982). The methodology applied in a QTL study to measure a trait should be objective and reproducible.
Rationale and significance

The production cost of grafted tomato seedlings is high. Currently, the price of a grafted seedling ranges from $0.90 to $1.12 per plant (Kubota et al., 2008; Rivard et al., 2010). The cost of grafted plants is due to the high cost of rootstock seed, the intensive work needed to graft, efficiency of the procedure relative to success, and post-graft care (Lee, 2003; Kubota et al., 2008; Lee et al., 2010; Rivard et al., 2010). My research addressed seed quality and graft success in an attempt to identify key steps where efficiency could be increased. Previous studies have indicated that rootstock germination is low (Smith, 1966) and physiological incompatibility between scion and rootstock add cost to the system (Lee and Oda, 2003). However, there are many potential benefits of grafting including increased yield, extended growth, disease resistance, and low and high temperature tolerance (Lee, 2003; Kacjan-Marsic and Osvald, 2004; Mohammed et al., 2009; Savvas et al., 2010; Rivard et al., 2010). Thus, addressing limitations to the production of grafted plants is desirable.

It is difficult to address some limitations due to industry trade secrets, inexperience with wild species used to produce rootstock, and the biology of grafting. Information about flowering conditions, day-length sensitivity, mating system, and the native habitat is available for many wild accessions (TGRC, 2012), but translating this information into a production setting will require effort. Remaining limitations include issues of seed quality and germination, and graft quality and efficiency (Ibrahim et al., 2001; Gisbert et al., 2011).
This research identified key restrictions in the process of developing new rootstocks and grafting rootstock to scion. Rootstock seed quality can be improved through a combination of genetic selection and manipulation of the seed production environment. Similarly, graft success can be improved through a combination of genetic and environmental manipulation. As a result of this research, I identified specific crosses with high seed quality. Similarly, I identified specific rootstock-scion combinations with high rates of success. I also demonstrate the improved success of grafting through use of cyanoacrylate adhesives. Finally, studies initiated to dissect the genetic basis of graft compatibility suggest a genetic component, but have been inconclusive at revealing the underlying genetic architecture.
Chapter 2 : Genetic and environmental factors affecting seed quality of tomato rootstocks using wild species

Abstract

Rootstocks are currently used in annual vegetable production to control soil-borne diseases, replace fumigation, and to impart vigor. The goals of this study were to evaluate the genetic and environmental factors affecting quality of hybrid tomato seeds. We developed a wide variety of hybrid tomato rootstocks based on cultivated parental lines as female parents (*Solanum lycopersicum* L.) and a range of wild species as male parents. Environmental effects were assessed through replicated experiments in two different seed production seasons (winter-spring) and (summer-autumn). Fruits were harvested at three maturation stages (breaker, full ripe and overripe) to evaluate the effect of fruit maturation on seed quality. Seed quality was assessed based on seed size and germination rates. During the experiments, fruit set and yield of seed were assessed. Our results show that seed production is affected by genetic components and environment, while seed quality showed higher genetic effect than environment. We found that fruit set rate was affected by maternal parent and environment (*P* < 0.05). Seed yield was determined by both parents and environment (*P* < 0.05). The paternal and maternal parents were a significant factor for determining seed size (*P* < 0.05) and seed
germination was determined by genetic components and environment factors. The effect of fruit maturation was significant on seed germination ($P < 0.05$). Larger seeds resulted in a higher germination rate. This study illustrates that both parents (female and male) contributed to seed quality for tomato rootstocks. In addition, our results suggest the possibility of selecting combinations of cultivated parental lines and wild species that produce high seed quality. The knowledge gained from this experiment will enable the selection and breeding of new tomato rootstock cultivars with multiple traits, which will improve the sustainability and profitability of both conventional and organic tomato production.

**Introduction**

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops in the world (Lucier et al., 2000). In the United States, between 2007 and 2012, tomato was planted on over 82,000 ha per year, with an annual crop value of approximately 1.8 billion dollars for the combination of processed and fresh-market crops (USDA-NASS, 2013). Based on economic value, fresh-market tomato is the most important fresh vegetable (USDA-NASS, 2013). In addition to its economic value, tomato provides vitamin C and pro-vitamin A (β-carotene) to the human diet (Rouphael et al., 2010). Therefore, per capita consumption of tomato has increased from 14.9 lb. to 17.8 lb. in the interval between 1985 and 2000 (Jones, 2012). Given projected population growth, it is necessary to continue to improve production efficiency for all crops while maintaining nutritional and market quality standards.
Tomato producers often face production problems related to soil-borne diseases and abiotic stresses that can reduce the yield and quality of fruit (Alam et al., 1994; King et al., 2008; Hasna et al., 2009; Cramer et al., 2011 and McAvoy et al., 2012). A number of methods are available to control soil-borne diseases, including: (1) host resistance (e.g., use of resistant varieties); (2) cultural control (e.g., crop rotation), (3) organic amendments, (4) physical control methods (e.g., solarization), (5) chemical fumigants (e.g., methyl bromide), and (6) biological control, which is under development (Schafer, 1999; Koike et al., 2003; McSorley et al., 2009).

Grafting is now a well-established technique in annual crops that has already shown promising results in diverse production environments. Grafting onto resistant rootstocks has the potential to address concerns about chemical use while increasing production efficiency (McAvoy et al., 2012). Furthermore, this technology is considered a component of Integrated Pest Management (IPM), which includes many other methods to control soil-borne diseases such as solarization (Ioannou, 2001), bio-fumigation, and intercropping (Kaskavalci et al., 2009). Grafting onto resistant rootstocks can be used to control soil-borne diseases (Harrison and Burgess, 1962; Bradley, 1968; Grimault and Prior, 1994; Paplomatas et al., 2000; Rivard et al., 2010; Barret et al., 2012 and McAvoy et al., 2012), increase yield (King et al., 2010), and impart tolerance to abiotic stresses (Black et al., 2003; Rivero et al., 2003 and Venema et al., 2008). Grafting can also avoid the loss of desirable traits and the time consuming processes associated with introducing resistance into contemporary cultivars. Many tomato rootstocks have been released to the market in recent years. However, only a few are widely used in practice because of the
superior performance or the availability of seeds in the market (Kubota et al., 2008; King et al., 2010). There are multiple reasons to increase the diversity of rootstock used by the industry. The overuse of the same rootstocks by growers may permit pathogen populations to evolve, resulting in the breakdown of resistance, as occurred in Japan with watermelon rootstock (Acquaah, 2007 and King et al., 2008). Currently, interest in breeding for improved rootstock is increasing rapidly in the private sector due to cost effectiveness and expanded disease resistance (King et al., 2010).

Rootstock seed quality is important because it affects seedling emergence, uniformity of growth, and diameter of the stem (Pet and Garretsen, 1983; Nieuwhof et al., 1989). Lack of uniformity creates inefficiencies and difficulties in the grafting process (Lee et al., 2010). Seed production and quality are often affected by genetic components, the maternal environment, and harvest time of the fruit (Baskin and Baskin, 1998; Contreras et al., 2008). Seed quality is measured by seed size, germination rate, genetic purity, freedom of contaminants, and moisture content. In tomato, the standards of high seed quality are genetic purity of 99%, germination rate above 75% (AASCO, 2013) or 85% (ISF, 2013), and moisture content less than 8%.

The use of F1 hybrids as tomato rootstocks has become common in tomato grafting. Early rootstock hybrids of tomato were produced using S. lycopersicum (formerly Lycopersicon Esculentum Mill) as a female parent and S. habrochaites (formerly Lycopersicon hirsutum var. ‘glabratun’) as a male parent (Bravenboer and Pet, 1962). Hybrid seed production from wide hybridization involves the fusion of the female and male gametes, where the aim of crossing is to combine important traits from the wild
species and the cultivated species. However, in some wide crosses reproductive barriers between parents affect the production of hybrid seeds. Crosses between cultivated tomato and its wild relatives, lead to vines displaying increased growth which is interpreted as increased vigor, and combine multiple traits (e.g. disease or insect resistance, salt tolerance, cold tolerance) (Rick, 1982; Rick et al., 1978; Thomas and Pratt, 1982; Rick et al., 1986 and Rick and Chetelat, 1995). At the same time, there are prezygotic and postzygotic interspecific crossing barriers between cultivated tomatoes and its wild relatives which may result in cross failure, seeds with low quality, or seed sterility in hybrids (Quiros, 1991; Rick et al., 1978 and Bedinger et al., 2011). The use of seed directly from wild species or from hybrid seed derived from wild species may, therefore, have a negative effect on seed quality.

To obtain high seed quality, environmental components of production and harvesting should also be identified and maximized. Ideally, seeds should be harvested when they reach physiological maturity (PM), which is when no additional assimilates are deposited in the developing embryo. However, there are discrepancies in the literature between and within species when defining PM. Some seeds achieve PM when the seed reaches the maximum dry mass (Harrington, 1977; Deluche, 1980) and others before they attain the maximum dry mass (Tekrony and Egli, 1997). Species where the seeds develop in fleshy fruits (tomato, peppers) may reach maximum quality after maximum dry mass is achieved (Valdes and Gray, 1998; Demir and Samit, 2001).

In recent years tomato breeders have been developing plant material with high lycopene content, which have health benefits because its antioxidant functions (Croteau
Plant material with high lycopene levels may present negatives effects on plant development including delayed germination or reduced plant growth, which was reported previously (Jarret et al., 1984; Berry and Bewly, 1992). Furthermore, high levels of carotenoids might inhibit the production of essential germination promoters including gibberellins (Fray et al., 1995), which will reduce the germinability of the seeds or delayed germination. Lycopene production is associated with fruit maturation (Arias et al., 2000). Therefore, it is essential to identify the ideal harvest time of tomato fruit for seed production.

To address the importance of genetic and environmental effects on rootstock seed quality, we evaluated a large number of wide crosses, with parents selected based on their potential contribution to resistance against biotic and abiotic stress. The objectives of this study were: (1) to quantify the influence of genetic and environmental factors on seed production and quality in hybrid crosses, (2) to assess quality relationships in order to define early selection criteria for rootstock breeding, and (3) to identify seed harvest time that maximize seed quality of hybrid tomato rootstocks.

**Materials and Methods**

*Plant material and growing conditions*

We obtained seeds from seven accessions of tomato and wild relatives from the Tomato Genetics Resource Center (TGRC) and another 13 accessions were used from our breeding program. Specific crosses were conducted using 10 inbred parental lines as female parents (*S. lycopersicum*), and 11 accessions of wild species as male parents (*S.*
*arcanum, S. chilense, S. corneliomulleri, S. habrochaites, S. pennellii, S. peruvianum, and S. pimpinellifolium*. The selection of these parents was based on traits such as resistance to soil-borne diseases and tolerance to abiotic stresses. Details are presented in (Table 2.1). All seeds were sown in 288-cell (Hummert, EARTH City, MO-USA) trays with a cell volume of 13 mL, using soilless mix PRO-MIX (Premier Hotticultural, Quakertown, PA – USA) and placed in a greenhouse. After four to five weeks, plants were transplanted into 3.78 L plastic pots (Hummert, EARTH City, MO-USA) using PRO-MIX.

In the greenhouse at the Ohio Agricultural Research and Development Center (OARDC) in Wooster, OH, day time temperatures were set between 27 °C – 30 °C and nighttime temperatures between 20 °C – 25 °C, with 12 h light and 12 h dark (long-day conditions). Plants were irrigated daily and uniformly fertilized with 250 mg l⁻¹ dose of 20N-20P-20K (Peters Professional All-Purpose Fertilizer, Scotts-Sierra Horticultural Products Co., Marysville, OH). Daily temperature data were obtained from the Ohio Agricultural Research and Development Center (OARDC) weather stations system (http://www.oardc.ohio-state.edu/newweather/dailyinfo.asp?id=1).

**Pollination**

All crosses were performed by hand pollination. Flowers from female plants were emasculated before flowers opened, typically a day before the anthesis. Pollen was collected from each male parent and placed on the stigma of the emasculated female flower. Each pollinated flower was labeled with a white tag containing the pollination
date and the parental information. The number of pollinated flowers per plant was 30–50 based on fruit set and flower production of the female plants. Non-pollinated flowers setting fruit were removed from female plants to avoid introducing selfed seed into evaluation of quality. Pollination took place over 1.5 months (experiment 1) and 3 months (experiment 2), generating 9 and 26 pollination dates for experiments 1 and 2, respectively.

Pollen collection was carried out for all wild male parents in bulk, and stored to maintain availability. Pollen was collected in a 0.2-mL eppendorf tubes by vibrating an open flower from the male plants. This collection was conducted during 2011 and 2012. Tubes containing pollen were placed into glass vials 50 mL (USA Scientific, Inc. Orlando FL) with full filled of drierite desiccant (W.A. Hammond Drierite Co., Xenia, Ohio) and stored at -80 °C until used (Sacks and Clair, 1996). On pollination day, the vial was removed from the -80 °C freezer 20-30 min before pollinating, warmed to room temperature and wiped dry to remove moisture before opening the glass vial and removing the eppendorf tube containing pollen.

The experiment was divided in two phases. In Phase 1, ten female and four male parents were used and the entire experiment was repeated twice: Jan. – Jun. 2012 and Jun. – Dec. 2012. Ten female plants were arranged in a randomized complete block design (RCBD). In the second phase, an experiment was conducted to compare 70 new crosses with two controls crosses used to normalize data using an augmented design. This second experiment involving these 70 new crosses was used to expand the genetic base of potential rootstock crosses using criteria identified from the first phase. The experiment
was conducted using an augmented randomized complete block design with four blocks consisting of 4 controls and 18 new crosses. Blocking was used to account for environmental gradients along rows and quadrants in the greenhouse.

Separate experiments were performed using a reduced number of genotypes in order to evaluate the effect of fruit harvest stage on seed production and quality. Four *S. lycopersicum* accessions were used as female parents (Hawaii 7998, Ohio MR13, FG02-188 and, Fla.7775). A partially inbred accession developed from LA2204 (*S. habrochaites*), LA2204B, and (*S. pimpinellifolium*) LA1589 were used as the male parents. These eight crosses were selected based on experience suggesting a range of seed quality (from poor to high) and on positive performance data of the resulting hybrids. A randomized complete block design with harvest time as a factorial with three replications was used. The experiment was repeated twice Jun. 2012 – Dec. 2012 and Dec. 2012 – May. 2013. Normally, the tomato seed producers harvest the fruit at breaker stage to full ripe, due to the high cost of maintaining the field for an extra time (Demir and Samit, 2001). In addition, over-ripe of fruit in mother plant increases lycope content, which may inhibit important germination promoters causing reduction in seed quality (Berry and Bewley, 1992). In this study our objective was to identify the best harvest time for wide-hybrid crosses. Thus, our main treatment was fruit maturity at harvest time: early (breaker stage), medium (full ripe), and late (over-ripe).

Harvesting of tomato fruit was carried out daily until the end of the season. A fruit was considered set if enlarged to > 0.5 cm diameter. Fruit set rate was determined as the total number of fruit divided by the total number of pollinated flowers on each plant.
Seed yield was determined as the total seed obtained divided by the total fruit harvested from each plant.

Seed extraction

Seed extraction was conducted by hand following the standard protocol used by the Tomato Genetics and Breeding Program, OARDC, Wooster, Ohio. All harvested fruit were cut in a half, and seed removed into a plastic beaker. 10% Hydrochloric acid (3 N HCl) was added to the seed and left standing for 30 minutes at room temperature (~ 25 °C). The seeds were rinsed under running tap water and placed back in the beaker. Trisodium phosphate (TSP) (10%) was added to the beaker containing seeds and soaked for 15 minutes. Then, seeds were rinsed again and dispersed on a screen. The screens with seeds were immediately placed in a dryer with air movement, and left overnight. The next day, the seeds were packed in coin envelopes and stored until the evaluation of seed quality.

For experiments aimed at measuring genetic effects, seeds were extracted three times a week during the peak of season and once a week toward the end of the seed production season. For the harvest timing experiment, seed extraction was done immediately or the day after harvesting the fruit.

Seed quality evaluation

We randomly pooled 200 seeds per hybrid and divided them into four subsamples (50 seeds). Seed size was determined by weighing replicates of these 50 seeds. The
average weights of the subsamples were used as an estimate of seed size. Germination tests were carried out according to International Seed Testing Association (ISTA) rules (ISTA, 2013). We evenly distributed 50 seeds in each Petri dish containing 9.0 cm × 2nm blue blotting paper (Anchor Paper, Co., St. Paul, MN) previously saturated with double distilled water (ddH₂O). The Petri dishes were then placed in a controlled environment chamber (Hoffman Manufacturing Inc. Albany, OR) at 25 ± 1 °C in the dark.

For the first replication of experiments designed to explore the relationship between fruit maturity and seed quality, germination test was extended for 7 d longer than recommended (ISTA, 2013) to enable more-dormant seeds to germinate. Germinated seeds were counted 7, 14 and 21 d after seeding. For the second experimental replication, the germination period was 14 d and germinated seeds were counted every day. For all experiments, newly emerged seedlings were removed from the Petri dishes, and seeds were regularly watered with double distilled water. Seeds were considered germinated when the radicle protruded from the seed coat. Seedlings were also evaluated as having normal or abnormal visual morphology.

Statistical analysis

Statistical analysis was performed using RStudio computer software version 0.97.320 (RStudio, 2012) in concert with the R core package version 2.15.3 (R Core team, 2011). In order to assess the relative contribution of genetic and environmental effects on fruit set, seed yield and seed quality data were treated as using a Random Effect statistical model in order to extract variance components using the Lme4 package.
(Bates et al., 2011). The statistical model contained all main effects and all possible two-way interactions between the factors.

The Agricolae package in R (De Mendiburu, 2012) was used to perform mean separation. Mean separations were based on Tukey’s test ($P < 0.05$). The Sciplot package was used to place error bars on the graphics (Morales et al., 2012). Seed size (weight) was related to germination with scatter plot, superimposed Locally Weighted Polynomial regression (LOESS) (Cleveland and Devlin, 1988) with second degree polynomial and the parameter alpha, controlling the size of the local neighborhood, set at 1 using the package ggplot2, with commands geom_smooth and stat_smooth, and overlapping two levels of confidential interval 95% C.I and 99.5% C.I, respectively (Wickham, 2009).

Data for the augmented design were analyzed based on heterogeneity within Blocks (Federer, 1961). The analysis of the data was performed using PROC GLM of SAS (SAS Institute Inc., Cary, NC, USA, 2010). This analysis takes into account variability amongst blocks, measured by the control hybrids. The values for new hybrid entries are adjusted based on the experimental model, allowing for comparisons of a larger number of hybrids in order to assess those that are worth selecting for further evaluation for potential as rootstock.

Results

Since the study was conducted in two season productions, air temperature data were collected from the OARDC weather system. In the winter-spring season (experiment 1), the daily maxima and minima air temperature ranged from -5.9 ºC – 33.7 ºC and -18 ºC – 20 ºC, respectively. For the summer-autumn season (experiment 2) the
daily maxima and minima air temperature ranged from \(-2.8 \, ^\circ\text{C} – 36 \, ^\circ\text{C}\) and \(-8.7 \, ^\circ\text{C} – 22 \, ^\circ\text{C}\), respectively.

Over 5,900 flowers were pollinated using 10 female cultivated genotypes and 4 male wild species to create 40 hybrids. These hybrids were evaluated for four seed production and quality traits including fruit set, seed yield (seeds/fruit), seed weight, and germination. Data were approximately normally distributed and analysis was done on untransformed data. Total variance for each trait was partitioned into the components associated with genetics (female and male parent), crossing season environment (experiment), the interaction between parents, and the interaction between parental genotypes by environment and block within environment (Table 2.2).

**Variance in seed production**

Seed production was highly influenced by both genetic components (female and male parent) and environment (seed production season). The maternal effect explained 16% of the total variation associated with fruit set. No significant effect was detected for male genotype on fruit set. For seed yield, an equal effect was identified for both parents, each contributing 14% to the total variation. The environmental effect was significant for both traits. Experiment to experiment differences explained 22% of the total phenotypic variation for fruit set and 27% for seed yield. The portion of variance explained by block within experiment was negligible for fruit set and 2% for seed yield. The variance explained by the interaction between parents was also low, 0% and 3% for fruit set and seed yield, respectively. No significant difference was detected for block within
experiment, or the interaction between parental genotypes, for either trait. The variance explained by the interaction between female genotype and the environment was 11% for fruit set and 8% for seed yield. The interaction between the male genotype and the environment explained 3% of the total variation for fruit set and 1% for seed yield. Uncontrolled error accounted for 47% and 30% of the total variation for fruit set and seed yield, respectively (Table 2.2).

Crosses in which Fla.7547, Fla.7775, VFNT Cherry, NC-HS-1 and Mogeor were used as a female parent the mean percentage of fruit set was above 50%. While, for H7998, UC-T338, FG02-188, H7997 and Ohio MR13 the rate of fruit set was below 50% (Figure 2.1-A). The lack of paternal (main) effects on fruit set suggests that this trait was mainly determined by maternal parent in wide crosses. The percentage of fruit set was 20% higher during the experiment 1 (winter-spring) than experiment 2 (summer-autumn) across all the female and male parents (Figure 2.3-A), suggesting an important role for environmental control during hybrid production.

Significant interaction was observed between female genotype × environment and male genotype × environment for fruit set (Table 2.2). The significant female genotype by environment interaction for fruit set was due to a change in rank and change in magnitude of the differences among the genotypes. Likewise, the significant male genotype by environment interaction for fruit set was due to change in rank among the genotypes. In experiment 1, hybrids formed using LA2204A were the highest seed producers. This rank changed for experiment 2, where hybrids produced with LA1589 were the higher seed producers. This result could be influenced by a temperature effect.
on the pollen viability of the *S. habrochaites* parent. In previous studies, heat tolerance was reported for *S. pimpinellifolium* pollen (Villareal et al., 1978; Abdul-Baki, 1991; Abdul-Baki and Stommel, 1995).

Seed yield ranged from 11 seeds/fruit (Ohio MR13) to 54 seeds/fruit (NC-SH-1) for female parents across male parents and environment (Figure 2.1-B). In contrast to fruit set, there was significant variation among paternal genotypes for seed yield (seeds/fruit). LA1589 (*S. pimpinellifolium*) resulted 31%, 43%, and 46% higher seed yield compared to LA2204B, LA2204A (*S. habrochaites*) and LA0716 (*S. pennellii*), respectively (Figure 2.2-A). The average yield of seed (seeds/fruit) was 43% higher during experiment 1 (winter-spring) compared to experiment 2 (summer-autumn) (Figure 2.3-B). This may be attributed to the extreme high temperatures occurred during the experiment 2. The female genotype by environment interaction was significant for seed yield. This significance was due to a change in the magnitude of the differences among genotypes between two experiments with minor changes in rank.

**Variance in seed quality**

The variances associated with genetic components were larger than the variance associated with environmental effects for seed size (seed weight) and seed germination (Table 2.2). The role of the female parent was highly significant for seed weight and germination, explaining 27% and 22% of the variance, respectively. The male parent was also highly significant for seed size and seed germination, contributing 57% and 17% to the total variance, respectively. The crossing season accounted for 13% of the total
variation for seed germination and none for seed weight. The female by male parent interaction was significant only for seed germination, contributing 26% to the total variation. Paternal genotype by environment interaction was significant, but explained only 2% and 3% of the variation for seed size and seed germination, respectively. We did not detect significant effects for female genotype by crossing season interaction and block within experiment interactions for either trait. Error accounted for 12% and 19% of the total variation for seed weight and germination, respectively (Table 2.2).

The seed weight for female parents ranged from 1.034 mg (VFNT cherry) to 3.14 mg (Fla.7775). This range indicates differences in the amount of resources allocated to the seeds among maternal parents. Such maternal effects on seed size are normal in plants (Roach and Wulff, 1987; Schwaegerle and Levin, 1990). Differences among paternal parents also contributed greatly to variation for seed size. For seeds resulting from pollinations with LA1589, the seed weight across female plants was 3.68 mg. Pollinations by LA2204B resulted in an average seed size of 1.97 mg, followed by LA2204A with 1.94 mg, and LA0716 with the smallest seeds at 1.42 mg (Figure 2.2-B). The ranks of seed weight by paternal parents were consistent across all the maternal plants. These ranks correspond to the genetic distance between the wild species, with S. pimpinellifolium most closely related to the cultivated types.

There was a significant male genotype by environment interaction effect for seed weight, although the magnitude of the interaction was relatively small in comparison to the main effect due to male genotype. This observation suggests that seed size is influenced more by male genotype than environment.
The germination for female parents across male parents and environment varied significantly, ranging from 34% (VFNT Cherry) to 83% (H7997) averaged across all crosses (Figure 2.1-D). Across female parents and environment for male parents the average germination varied from 49% (LA2204A) to 85% (LA1589) (Figure 2.2-C). In experiment 1, conducted in the (winter-spring) season the percentage of seed germination was higher 74%, compared to experiment 2, conducted in the (summer-autumn) season, which averaged 59% of final germination across parental genotypes used in the study (Figure 2.3-C).

The seed germination was also significantly affected by female and by male parent interactions, revealing genetic variation among hybrids for germination response (data not shown). Of ten female parents used in this study, eight had high germination when hybrids were formed with LA1589. Seven had high germination when crossed to LA0716. Four females had high germination rates when crossed to LA2204B, and only one had high germination when crossed to LA2204A. Variation was also detected for germination based on female parents. The multi-resistant female parent Mogeor had relatively high seed germination across all male parents used in pollination. The germination percentage was low for Ohio MR13 female parent independent of male parent used as a pollen donor.

The effect of paternal parent by crossing season interaction was significant, which was due to change in the magnitude of differences among paternal genotypes between experiments. Seeds from experiment 2 pollinated with LA2204A had lower germination rate compared to experiment 1 and other male parents within experiment 2.
**Seed weight and germination**

We wanted to know if selection for rootstock seed could be performed before germination tests, without negatively influencing which hybrids might be carried forward in a breeding program. The mean of the final germination percentage was plotted over the mean of seed weight, a curve fit to the data, and confidence intervals of 95% and 99.5% were superimposed (Figure 2.4). Seed weight ranged from 0.04 mg to 4.56 mg with the large differences in seed weight among the hybrids also reflected in wide differences in germination which ranged from 15% to 97%. Germination from large seeds was better than from intermediate and small seeds (Figure 2.4). These results indicate that selection is possible based on seed weight, thus avoiding the need to perform germination tests for clearly inferior material.

In the second phase, an experiment was conducted to compare the genetic potential of 70 new crosses. Control crosses were replicated in each block, in order to correct for spatial variation. The controls were chosen based on evaluation during phase 1. Using this approach, we were able quickly to evaluate a larger number of crosses, using available space, time, and human resources. The results for seed production and quality of the 70 new crosses and the 2 controls are shown in Table 2-3. Least squares means for fruit set across hybrid ranged from adjusted values of -11% to 98%. Seed yield ranged from adjusted values of 1 seed/fruit to 117 seeds/fruit. Twenty-four hybrids were dropped from seed weight and seed germination tests due to low seed number.

Based on seed weight and germination, of the 70 new hybrids, only 11 would be worth pursuing as potential rootstocks. FG12-501 to FG12-510 were crossed with
LA2533 (*S. pimpinellifolium*), FG12-511 to FG12-520 with LA2157 (*S. arcanum*), FG12-521 to FG12-530 with LA 1929 (*S. peruvianum*), FG12-531 to FG12-540 with LA1937 (*S. cornelioniulleri*), FG12-541 to FG12-550 with LA1959 (*S. chilense*), FG12-551 to FG12-560 with LA2931 (*S. chilense*), and FG12-561 to FG12-570 were crossed with LA0407 (*S. habrochaites*). The seed weight among the hybrids ranged from 0.001 mg (FG12-520) to 4.56 mg (FG12-507), and seed germination varied from -7% (FG12-516) to 93% (FG12-504) (Table 2-3). Based on these results, only LA2533 (*S. pimpinellifolium*) and LA0407 (*S. habrochaites*) produced any hybrids with seed quality that would warrant further evaluation and optimization (Table 2.3).

In order to investigate the potential for improved seed quality, we investigated the effect of fruit maturation on seed germination. Selected parents were used for this study H7998, OhioMR13, FG02-188, and Fla.7547 as female parents and LA2204B and LA1589 as male parents. Harvest of fruit was timed to correspond to breaker stage, fully ripe and over ripe. Time of harvest was significantly associated with germination rates ($P = 0.003$). Seed germination for seeds harvested at full ripe stage was higher compared to breaker stage for most of the hybrids. However, we detected differences among hybrids for germination and germination speed. Seed of the hybrids FG02-188 × LA1589 and FG02-188 × LA2204B were less affected by fruit harvested at the breaker stage than the other six hybrids (Figure 2.5-A). From the results of our study, FG02-188 × LA1589 and FG02-188 × LA2204B hybrids can be harvested early and high seed quality can be obtained. For the crosses, OhioMR13 × LA2204B, Fla. 7547 × LA2204B, OhioMR13 × LA1589 and Fla.7547 × LA1589 final germination was significantly increased when
fruits were harvested at the overripe stage. The wide hybrid FG12-604 was limited by genetic compatibility between the female (H7998) and male (LA2204B) parents (Figure 2.5-C).

Discussion

High seed production and quality are important in hybrid seed production to justify the extra cost added by emasculation and pollination, which is labor intensive, and therefore, expensive (Georgiew, 1991; Opeña et al., 2001).

In the present study, seed quality was the product of interactions between environmental and genetic components (female and male parents). While seed size was maintained between environments (experiment), seed germination was higher during winter-spring season (experiment 1) compared to summer – autumn season (experiment 2). This may due to the extreme high temperatures occurred during experiment 2, which may cause dormancy on some hybrids or abnormal germination. In addition, we identified that seed size is an important predictor for seed germination at early selection point.

There was a wide variation in fruit set and seed yield, which can be attributed to a maternal parent by environment interaction. These results are not surprising given that maternal parent determines the number of ovules, provides resources to the new embryo and produces the seed coat (Roach and Wulff, 1987).

Tomato fruit set is optimal between 17 - 18 °C night and 20 – 25.6 °C d (Went, 1944; Leopold and Scott, 1952; Charles and Harris, 1972). According to the NOAA’s
report, the summer of 2012 was the third hottest summer on record for the United States. Extreme climate conditions could lead to overheat the greenhouse during summer time and to expose the plants to heat stress. Cooling a greenhouse in a hot summer is difficult and complicated task compared to heating a greenhouse during wintertime (Nilsen et al., 1984; Baille et al., 1994; Baille and Leonardi, 2000; Kittas et al., 2005).

The low fruit set and seed yield obtained in experiment 2 (summer-autumn season) can be attributed to high temperature in the greenhouse, which is well documented that extreme temperatures has negative effects on yield (Alam et al., 2010), and reproductive organs. High temperature can affect fruit set and seed yield by affecting meiosis in the pollen and ovule mother cells, development of the endothecium in the anther, then reducing the dehiscence and pollen shed, inhibiting pollen germination, slowing pollen tube growth, drying the stigma surface and causing low pollen retention, reduce growth of the endosperm pre-embryo and early stage zygote. Reducing the growth on fertilized embryo could result in small undeveloped seeds, which could be lost during seed extraction process (reduce seed yield) (Iwahori, 1965, 1966; Picken, 1984; Dinar and Rudich, 1985; Dane et al., 1991; Pet et al., 1997; Hanson et al., 2002; Sato et al., 2000; Max et al., 2009). Thus, it is important to maintain the ideal temperature in order to obtain high seed yield when producing hybrid seed tomatoes.

These results suggest that the ideal season for seed production under controlled environment is winter-spring season. In addition, selecting both female and male parent based on their response to environment seed production can be improved under harsh environment conditions.
Based in the results we conclude that seed size is determined mainly by paternal genotype, contradicting previous studies (Pet and Garretsen, 1983), where they found that seed size is mainly determined by maternal genotype. These differences might be due that they used all inbred lines (female and male parents), while in this study we produced wide hybrid seeds using wild relatives, where paternal parent had stronger influence. Therefore, when using wild relatives it must be keep in mind that seed size can either increase or decrease.

Marked differences due to genetic components were obtained for seed germination. In general, the genetic distance between female and male parent can predict seed germination, but some wide crosses that can perform the same or better than crosses with short genetic distance parents. In this study we identified seven wide hybrids crossed with LA0716 (S. pennellii), three with LA2204B (S. habrochaites) and only one with LA2204A (S. habrochaites) that reached the minimum national seed germination standard 75% (AASCO, 2012).

Data presented here (Figure 2. 4), out of 40 plotted germination points, the six smallest hybrids showed the lowest germination rate. Thus, selection could be performed before the germination test by cutting off at 1.4 mg. of seed weight. At this selection point, we could be omitting 33% seeds with low quality and retaining a 100% of high quality seeds for germination test. This selection point can be increased to 1.8 mg. where 43% of seeds with low quality can be omitted and retained 74% of high seed quality for seed germination test. In the extreme scenarios, we can draw a line at 2.2 mg. for seed
germination where 62% of low seed quality can be omitted and retained only 53% of high seed quality.

The effect of seed weight on final germination may be due to variation in food reserves in the seeds and influenced by the interaction of the parents. H7998 and VFNT Cherry resulted in the lowest seed weight and final germination when they were crossed with LA2204A, LA2204B and LA0716. In contrast when crossed with LA1589, the seed size increased up to 90% for H7998 and 88% for VFNT Cherry. The final germination also increased up to 80% and 83% for H7998 and VFNT Cherry, respectively.

Part of the seed weight effect on seed germination seemed to be parentally determined, since the hybrids crossed with S. pimpinellifolium showed higher seed weight and high seed germination. Hybrids crossed with S. pennellii, even the seed weight was lowest the germination was the second highest compared to other male parents. The hybrids crossed with S. habrochaites had the lowest seed germination but seed weight was the second largest compared to other male parents.

Hybrids crossed with the two partially inbred selection S. habrochaites showed a correlation in seed size where LA2204B had 22% higher seed germination and 2% higher seed weight compared to LA2204A. This result indicates that these two male parents are different between each other, due to selection over the time in the greenhouse.

Previous studies looked at the correlation between seed size and time to germinate (Whittington et al., 1965). In this study, we evaluated the association of seed weight and final germination percentage. The effect of seed weight on germination could be due to
food reserve, which can be influenced by the maternal environment during seed production.

The maximum dry weight of tomato seeds is attained before physiological maturity, which is at breaker stage (Valdes and Gray, 1998; Demir and Samit, 2001). In this study, over the range of fruit ripeness from breaker stage to overripe no changes were detected in seed weight. This result indicates that wide hybrid crosses in tomato reaches the maximum mass at the same time as self-seeds breaker stage (Valdes and Gray, 1998; Demir and Samit, 2001). However, maximum seed quality in terms of seed germination is not obtained at breaker stage for the majority of hybrids.

Fruit maturation can be one of the factors to manipulate in order to increase seed quality, but not all hybrids can be improved. Seeds from fruits harvested at breaker stage had lower final germination; it could be due to high percentage of immature seeds. The majority of the hybrids produced in this study showed higher seed germination when the fruits were left on the plant mother until overripe stage. While, only two out eight hybrids resulted to have high germination when harvested at breaker stage, this seems to be maternal determined (FG02-188).

The female parent FG02-188 crossed with both male parents showed a higher final germination regardless of the harvesting time. However, there was higher speed germination when fruits were harvested at overripe stage. In general, the germination speed was higher in seeds from fruits harvested at overripe stage (Figure 2.5-A-B-C) and has a genetic component, which was observed before (Demir and Ellis, 1992; Berry and Bewley, 1992).
Fruit maturation can affect the final seed germination and speed germination in wide hybrid seeds, both traits are important for seed industry. These findings indicated that wide crosses could produce rootstocks with high seed quality. In addition, fruit maturation a key point to manipulate in order to increase seed quality in wide hybrids. At the same time, control of the seed production environment is essential for better quality seed production in wide-cross hybrid tomato seeds. Finally, we identified key selection points in the breeding of new tomato rootstock cultivars.
### Tables

<table>
<thead>
<tr>
<th>Accession</th>
<th>Variety Name</th>
<th>Source</th>
<th>Parent</th>
<th>Species</th>
<th>Resistance/tolerance</th>
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<td>TGRC</td>
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<td>S. lycopersicum</td>
<td>Frl, I, I^2, Mi, pyI, Tm2^2, Ve</td>
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<td>OHIO</td>
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<td>OHIO</td>
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<td>Phytophthora infestans Xanthomonas gardneri</td>
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<td>Male</td>
<td>S. penellii</td>
<td>I^3, Xanthomonas campestris, Alternaria alternata, salt, drought</td>
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*FG02: Tomato Genetics Resource Center, Department of Vegetable Crops, University of California, Davis, CA 95616, USA; OHIO: Tomato Genetics and Breeding Program, Department of Horticulture and Crop Science, Ohio Agricultural Research and Development Center - The Ohio State University, Wooster, Ohio 44691, USA.

**Table 2.1:** Female and male parents used in hybrid seed production.
<table>
<thead>
<tr>
<th>Source of variance</th>
<th>Fruit set (%)</th>
<th>Seed yield (Seeds/fruit)</th>
<th>Seed weight (mg)</th>
<th>Germination (%)</th>
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<tr>
<td></td>
<td>Estimate</td>
<td>% of variance</td>
<td>Estimate</td>
<td>% of variance</td>
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<td>0.004</td>
<td>16***</td>
<td>96.21</td>
<td>14***</td>
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<tr>
<td>Male parent y</td>
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<td>0NS</td>
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<td>14***</td>
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<td>Female:Male</td>
<td>0.000</td>
<td>0NS</td>
<td>21.97</td>
<td>3NS</td>
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<td>Environment x</td>
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<td>177.87</td>
<td>27***</td>
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<tr>
<td>Male:Env</td>
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<td>3*</td>
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<td>Error</td>
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<td>47</td>
<td>202.43</td>
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</table>

NS, *, **, *** Nonsignificant or significant at P ≤ 0.05, ≤ 0.01 and ≤ 0.001, respectively.

\(^z\) Ten inbred parental lines (S. lycopersicum) were used as female parents to evaluate fruit set (%), seed yield (number of seeds per fruit), seed weight (mg) and seed germination (%). Females were Mogeor, UC-T338, VFNT Cherry, Fla. 7547, NC HS-1, Ohio MR13, Fla. 7775, FG02-188, Hawaii 7998, and Hawaii 7997.

\(^y\) Four wild accessions were used as male parents LA1589 (S. pimpinellifolium), LA0716 (S. pennellii) and two partially inbred accessions developed from LA2204 (S. habrochaites), LA2204A and LA2204B.

\(^x\) Experiments were repeated two times, January – June 2012, and June – December 2012 to evaluate the effects of environment.

Table 2.2: Estimated variance components for female parents, male parents, experiment, and interactions on fruit set, seed yield, seed weight and seed germination.
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<th>SW (mg)</th>
<th>SG (%)</th>
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</table>

C-1: control-1; C-2: Control-2; X: Absence of test due to low seed or zero; FS: Fruit set; SY: Seed yield; SW: Seed weight; SG: Final germination.

**Table 2.3:** Mean comparisons using Least Squares Means with adjustment for multiple comparisons using Dunnett’s test and control C-2 for evaluation of hybrid seed quality.
Figure 2.1: Effect of female parents on (A) Fruit set (%), (B) Number of seeds per fruit, (C) Seed weight (mg), and (D) Seed Germination (%).

Bars represent SE (n = 16). Letters above the SE bar are based on Tukey’s mean separation, when the same letter is present there is no significant difference at $P \leq 0.05$. 
Figure 2.2: Effect of male parents on (A) Number of seeds per fruit, (B) Seed weight (mg), and (C) Seed Germination (%).

Bars represent SE (n = 40). Letters above the SE bar are based on Tukey’s mean separation, when the same letter is present there is no significant difference at $P \leq 0.05$. 
Figure 2.3: Effect of Environment (Experiments) on (A) fruit set (%), (B) Number of seeds per fruit and (C) Seed germination (%).

Experiment 1 was conducted January-June 2012 and Experiment 2 was conducted June – December 2012. Data represent the means and error bars indicate ± SE of two independent experiment, ten female and four male parents (n = 80). Letters above the SE bar are based on Tukey’s mean separation, when the same letter is present there is no significant difference at $P \leq 0.05$. 

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Figure 2.4: Relationship between seed size (weight) and seed germination.

Data for 40 hybrids were used to fit a curve line and superimpose confidence interval (shaded). Germination of 75% (horizontal line ------) and 85% (horizontal line -- - - - -) are considered the legal minimum standard (AASCO, 2012; ISF, 2013) respectively.
Figure 2.5: Germination curves for seeds from eight tomato hybrids harvested at different stages of fruit maturation.

The small vertical bars represent SE for each point. In A: Fruits were harvested at the breaker stage. In B: seeds were extracted from fruits at the full ripe. In C: seeds were extracted from overripe stage fruits. Hybrids FG12-601 to FG12-604 the pollen donor is LA2204B and FG12-605 to FG12-608 the pollen donor is LA1589. Germination of 75% (horizontal line———) and 85% (horizontal line………………) are considered the legal minimum standard (AASCO, 2012; ISF, 2013) respectively.
Chapter 3: Cyanoacrylate adhesive improve grafting success between tomato rootstock and scion

Abstract

Grafting annual fruits and vegetables can improve crop yield and increase disease resistance; however, the process is labor intensive and economically unviable for most crops. We studied an alternative method to increase grafting success of annual vegetables using tomato as a model. Nine rootstocks were used, including accessions of the wild species Solanum pimpinellifolium (LA1589) and S. habrochaites (LA2204). We also included a processing tomato S. lycopersicum (FG02-188), an unimproved variety Hawaii 7998 (H7998), hybrids derived from crossing these parents (SGH07-315, SGH07-316, SGH07-320, SGH07-326), and a commercial rootstock ‘Maxifort’. The experiment was repeated five times. Tube grafting, a widely used method, was compared to grafting using ethyl-2-cyanoacrylate adhesive. The scion used was Cherokee purple, an heirloom variety. We also evaluated survival rate in open field studies and yield data was collected from two rootstocks. Grafting success was significantly affected by the methods and rootstock genotypes ($P < 0.05$) in the greenhouse study. Using the adhesives method resulted in a better survival rate. Rootstocks Maxifort and SGH07-315 showed higher grafting success over all the experiments and grafting methods. Success for H7998 was low, suggesting that this parent contributed to poor scion-rootstock compatibility.
In the open field study, grafting success was affected by rootstocks ($P < 0.05$) and no differences were observed between grafting methods. Yield data revealed that using adhesives in the grafting process did not have negative effects. Grafting success was dependent on the choice of rootstock and could be improved by using adhesives.

**Introduction**

Grafting of annual crops has been used in Japan and Korea since the late 1920s. Grafting of annual fruits and vegetables was first documented for watermelon (*Citrullus lanatus Matsum. et Nakai*) using pumpkin (*Cucurbita spp.*) rootstock to provide resistance to Fusarium wilt (*Fusarium oxysporum*) (Tateishi, 1927). In the United States, small farmers grafted vegetables onto weeds in small scale in the middle of 1940’s (Kubota et al., 2008). Later the technique was applied to the production of cucumber and tomato (Lee, 1994; Oda, 1995, 1999 and 2002). In the early 1960s, grafting was introduced into commercial production of tomato in many parts of the world (Harrison and Burgess, 1962; Bravenboer and Pet, 1962; Upstone and Finney, 1966 and Lee and Oda, 2003). This technology was primarily introduced into annual crops to control soil-borne diseases caused by bacteria, fungi, and nematodes. Production of annual horticultural crops using grafted seedlings has become a common practice in many parts of the world, especially in Asia and parts of Europe where land use is intensive. Grafting also dominates hydroponic production environments in North America (Lee, 1994; Oda, 2002; Lee and Oda, 2003; Kubota et al., 2008). In the United States and other western countries where land use is not intensive, grafting is less common for soil-based
production systems. Nevertheless, grafting is becoming more widely used because of the benefits in resistance, vigor, and increase production (Lee, 1994; Fernandez-Garcia et al., 2003; King et al., 2010; Rivard and Louws, 2008; Savvas et al., 2009).

Interest in grafting Solanaceous plants has increased in recent years due to promising results such as resistance to a wide range of pathogens in the soil, tolerance to abiotic stress, and increased yield and fruit quality (Oda, 2002; Lee, 2003; Kubota et al., 2008). Grafting may provide a chemical free solution to tomato production (Miguel et al., 2004; Pogonyi et al., 2005; Rivard and Louws, 2008; Kubota et al., 2008; King et al., 2010). The harvesting season could also be extended using grafted plants in vegetables (Lee, 2003). Adoption of rootstocks accelerated when interspecific crosses exhibited a high level of resistance to *Pyrenochaeta lycopersici* (corky root rot) were introduced (Lee, 1994; Oda, 1995). It was later observed that these hybrids also had increased cold tolerance and high vigor (Lee, 1994; Oda, 1995). In 1996, resistance to *Fusarium oxysporum f. sp radicis-lycopersici* (FORL) (crown root rot) was added to the rootstock resistance profile. Later, seed germination was improved by commercial seed treatments, and the rootstock market grew to its current size (King et al., 2010). Introduction of the ‘Japanese” (tube) grafting method to Europe led to significantly lower the cost of grafting in large scale (Kurata, 1994; Lee, 1994 and Oda et al., 1994)

For tomato, there are two common grafting methods; cleft grafting and tube grafting (Lee, 1994; Oda, et al., 1994; Oda, 1995 and 1999). For the cleft grafting method, the stem of the scion is cut in a wedge, and this tapered scion is fit into a cleft cut in the end of the rootstock. The graft is then held firm with a plastic tube or clip (Oda,
Using cleft grafting method, the success of grafting is high and plants with different stem diameters can be matched excellently. The disadvantage of the cleft graft method is that the grafting process requires multiple cuts and is therefore labor intensive. Another method widely used in Korea and Japan is tube grafting. Tube grafting makes it possible to use younger plants; therefore, more plants can be grown per plug tray, and the grafting process is two to three times faster than with cleft grafting (Oda, 1999). Smaller plants also require less space in healing chambers or acclimation rooms. For this method, the rootstock and scion are cut once at the same angle. The cut ends are then joined by a tube or silicon clip, splicing the cut surface together (Oda, 1999). This method is potentially faster, but it requires the diameter of the scion and rootstock to be well matched. In our experience, the failure rate is higher with tube-grafted plants.

Problems associated with grafting remain to be addressed before the technique will be widespread for soil-based production systems. Grafting is labor intensive, which adds additional cost to the production system (Lee, 1994; Leonardi and Romano, 2004). This cost can be magnified when incompatibility between scion and rootstock limits the rate of survival (Davis et al., 2008). Despite of favorable reports of grafting, reductions in yield and fruit quality have also been documented due to incompatibility between the scion and rootstock (Edelstein, 2004; Kacjan-Marsic and Osvald, 2004). An increased survival rate is important to compensate for the additional costs of grafting. Therefore, new methods should be explored in order to take full advantage of this technique.

The use of cyanoacrylates as a surgical closure for wounds (Andrade et al., 2001; Albuquerque et al., 2006 and Endo et al., 2007), and the related adhesive, histocryl, for
aesthetic facial plastic surgery (Kamer and Joseph, 1989) suggest their potential use in other biological systems to join living tissue. The wide use of cyanoacrylate adhesives is facilitated by the low price and ease of use. Grafting eggplant, cucumber, Chinese cabbage and grapevine is facilitated using adhesives (Oda, 1995; Lee and Oda, 2003). The objective of this study was to increase the success rate of grafting using ethyl-2-cyanoacrylate adhesives (Krazy Glue; Super glue) for tomato grafting. The aim of using adhesives such as ethyl-2-cyanoacrylate is to simplify the procedure and improve the efficiency in grafting tomato.

**Materials and methods**

*Plant material and growing conditions*

We used nine tomato genotype rootstocks including two wild species LA1589 (*S. pimpinellifolium*), an inbred selection from LA2204 (*S. habrochaites*), one processing material FG02-188 (*S. lycopersicum*), H7998 (Hawaii 7998 an unimproved); the hybrids SGH07-316, SGH07-315, SGH07-320, SGH07-326; and ‘Maxifort’ as a control for all experiments. The scion was ‘Cherokee Purple’, an heirloom variety.

Experiments were conducted in the greenhouse facilities at The Ohio State University, Ohio Agricultural Research and Development Center (OARDC), Wooster OH. Sowing times of the scion were staggered over three weeks to provide a variety of stem diameters to match with the rootstock at the time of grafting. One week before the rootstocks, at the same time as the rootstocks, and one week after the rootstocks were seeded. All seeds were planted in plastic plug 288-cell (Hummert, EARTH City, MO)
trays with a cell volume of 13 mL (Hummert, EARTH City, MO) using PRO-MIX (Premier horticulture, Quakertown, PA - USA). Twenty-five days after seeding, the rootstocks were transplanted into 40-cell trays with cell volume of 68 mL (Hummert, EARTH City, MO) using PRO-MIX. Temperatures were set at 24/18 °C day/night and a 12-h photoperiod. Fertilization was applied using 20N-20P-20K (Peters Professional All-Purpose Fertilizer, Scotts-Sierra Horticultural Products Co., Marysville, OH) with every irrigation time; plants were irrigated one or twice a day.

**Grafting process**

Seven days following transplanting, rootstocks were grafted using tube grafting (Japanese top-grafting; Oda, 1999, 2002) or a modification using ethyl-2- cyanoacrylate adhesive (Krazy Glue, Columbus, OH). Both rootstock and scion were cut at 35° angles. The scion was aligned with the rootstock to ensure contact vascular tissue between the two plants. Tube grafting followed procedures described previously (Oda, 1995; Oda, 2002 and Rivard and Louws, 2008), with clips replaced by Tygon tubing (1.5 mm and 2 mm) cut to 1 cm length and slit down one side. The ethyl-2-cyanoacrylate grafting followed the same steps, but replaced the Tygon-tubing clip with a brushing adhesive to the outside of the graft. Care was taken to avoid introduction of adhesive between the cut surfaces of both plants.
Healing conditions

The new grafted plants were placed in a 5.5 m × 1.2 m × 0.7 m dimension healing chamber constructed from 0.75-inch PVC pipe as a frame and 0.08-mm plastic covering to maintain high humidity (95% - 100%). The greenhouse bench was covered with a capillary mat to keep constant humidity (Agriculture solutions, Columbus, OH), which was continually saturated with water. Shade cloth (60% shade) was used to cover on the plastic for the first week to minimize water loss of the new seedlings through transpiration. Temperatures in the greenhouse were set at 30 °C day/25 °C night and no artificial light was provided during the healing period. Seven days after grafting, the shade cloth was gradually removed by rolling up the sides, and the healing chamber was opened to increase light and lower humidity. Fourteen days post-grafting, the shade cloth, and plastic were completely removed.

Experimental Design

The experiment was conducted in a randomized complete block design with block replication occurring over time. These experiments were conducted at different seasons of the year including late autumn (Experiments 1 and 2), spring (Experiment 3) and late spring (Experiments 4 and 5). Rootstock genotypes were randomized in each of five experiments (blocks). Genotypes H7998, LA2204 and SGH07-316 were omitted from the second experiment because poor germination of seed prevented sufficient replication. The treatments included tube grafting and adhesive grafting. Twenty-five plants per rootstock were used for each treatment in each of the five experiments. Data collection
began one week post-grafting and continued for three weeks. Grafting success was evaluated by determining the number of grafted plants that survived (Alive) and showed a growth of the leaves at week one, two and three.

*Field experiment*

An open field experiment was carried out in Wayne County, OH (long. 40°46′46″N, lat. 81°55′30″W) to determine survival rate (Alive plant at harvesting time) and productivity of grafted plants using adhesives and tube grafting. From the last greenhouse experiment, late spring (Experiment 5), six rootstocks were selected for the field study based on the plant availability and the results of greenhouse experiment. The rootstocks chosen were ‘Maxifort’ and SGH07-315 (with high grafting success), LA1589 and FG02-188 (with medium grafting success), and LA2204 and H7998 (with low grafting success) at week 3 in greenhouse conditions. In the field trials, the two grafting methods for each rootstock genotype were compared. Each rootstock genotype-grafting method consisted of four plants per plot in a randomized complete block design with three replications. Transplants were set into a raised-bed plasticulture system. Plants were irrigated as necessary. Plant spacing was set at 61 cm within the row and 3.4 m between rows. Harvest was carried out on 21, 28 Sept. and 11 Oct. 2012 for ‘Maxifort’ and SGH07-315 rootstocks to determine the effect of the grafting method on total productivity. Total fruit per plant was weighted for each combination (‘Maxifort’ rootstock grafted using adhesives and tube grafting method; SGH07-315 rootstock grafted using adhesives and tube grafting methods).
Statistical analysis was performed using the statistical package in “Rstudio” computer software version 0.97.320 (RStudio, 2012) in concert with the R core package version 2.15.3 (R Core team, 2012). The Agricolae package (De Mendiburu, 2012) was used to perform mean separation. The Sciplot package (Morales et al., 2012) was used to place error bars on the graphics. Data were analyzed using analysis of variance (ANOVA) with main effects and all possible interactions between the factors. Differences between grafting methods and genotype were assessed by least significant differences (LSD) test at $P = 0.05$ after a significant F-test in the ANOVA. A fixed effects model was used to estimate LSMEANS based on a linear model. Mean values were separated by paired t-test for survival rate of grafting method for each rootstock genotype at $P < 0.05$. The following model was used to test the effect of grafting using adhesives on grafting success at week 1, week 2 and week 3 post-grafting:

$Y_{ijk} = \mu + T_i + G_j + E_k + TG_{ij} + GE_{jk} + TE_{ik} + \varepsilon_{ijk}$

Where $Y_{ijk}$ was the grafting success data, $\mu$ was the overall mean, $T_i$ was the effect of grafting methods, $G_j$ was the effect due to rootstock genotype, $E_k$ was the experiment effect. The interactions terms were $TG_{ij}$ was the effect due to grafting method by rootstock genotype interaction, $GE_{ik}$ was the effect of interaction between rootstock genotype x experiment, $TE_{ik}$ was the effect due to grafting method x experiment interaction, and $\varepsilon_{ijk}$ was the experimental error.

Normalization of the data was accomplished within our fixed effects model by estimating LSMEANS. In replicate 2, data were missing for genotype H7998, LA2204
and SGH07-316. Due to missing data, LSMEANS for each genotype, experiment, and treatment were extracted using the fixed effects model:

\[ Y_{ijk} = \mu + TGE_{ijk} + \varepsilon_{ijk} \]

Where \( Y_{ijk} \) was the grafting success value (%), \( \mu \) was the overall mean, \( TGE_{ijk} \) was the interaction between grafting method, rootstock genotype and experiment, and \( \varepsilon_{ijk} \) was the experimental error. Analysis of variance was repeated using LSMEANS values to verify that our results were not influenced by missing data.

**Results**

We grafted over 2245 plants; representing nine tomato rootstocks with one common scion, using two grafting methods and repeated the experiment five times. High variation was observed among the experiments. Late autumn grafting success across rootstock ranged from 4% to a maximum of 88% (Exp. 1 and 2), spring grafting ranged from 19% to 100% (Exp. 3) and late spring ranged from 25% to 100% (Exp. 4 and 5). Success for grafting the control ‘Maxifort’ ranged from 52% to 100% (using adhesive) and 16% to 80% (using tube grafting). This high variation between experiments was attributed to the seasonal weather variation. The air temperature, light, and humidity might have an influence on the greenhouse environment and subsequently on the healing chamber. The air temperatures ranged from 18 °C to 25 °C (Exp. 1 and 2), 25 °C to 27 °C (Exp. 3) and 24 °C to 28 °C (Exp. 4 and 5). The total natural light ranged from 37 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) to 397 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (Exp. 1 and 2), 31 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) to 823 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (Exp. 3) and 42 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) to 187 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (Exp. 4 and 5). The Relative humidity of the air
ranged from 17% to 61% RH (Exp. 1 and 2), 27% to 59% RH (Exp. 3) and 37% to 70% RH (Exp. 4 and 5). Despite the observed variation, there were significant differences observed for the main effects of rootstock genotype and grafting method (Table 3.1).

Rootstock genotypes

There were significant differences \( (P = 0.0126) \) among the rootstock genotypes for grafting success on the first and final week (week 1 and week 3) (Table 3.1). The rootstocks Maxifort (commercial variety) and SGH07-315 (hybrid) exhibited higher rates of success across all experiments at three weeks. Hawaii 7998 (H7998), LA2204 and SGH07-326 had the lowest rate of grafting success, with < 50% success observed at the final week (Table 3.2; Figure 3.1). No differences existed between rootstock genotype for grafting success on the second week (week 2) (Table 3.2).

Grafting methods

There were also significant differences \( (P < 0.0001) \) in grafting success between grafting methods (Table 3.1). Across the five experiments and nine rootstocks, the survival success was higher using adhesives, compared to tube grafting over three weeks (Figure 3.2). There were no significant interactions between grafting methods and rootstock genotypes (Table 3.1). In addition, the grafting success was higher than 50% for seven out of nine rootstocks using adhesive. On the other hand, only ‘Maxifort’ and SGH07-315 survived above 50% using the tube-grafting method across all the experiments (Figure 3.3).
Field experiment

Survival in the field was unaffected by grafting method ($P = 0.131$), but was affected by the rootstock genotype ($P = 0.012$). Maxifort, and SGH07-315 rootstocks had the highest survival rates of 100% and 96%, respectively. The H7998 genotype rootstock had the lowest survival rate of 49% in the field study. Even though, no statistic differences were identified in yield between grafting methods and rootstock genotypes ($P = 0.131$; $P = 0.134$, respectively), a numerical differences were detected between grafting method for both rootstock evaluated. The commercial rootstock ‘Maxifort’ had the highest yield per plant (14 kg•plant$^{-1}$) when grafted using adhesive compared to only (11 kg•plant$^{-1}$) using tube grafting method. The hybrid rootstock SG07-315 showed a similar trend of (12 kg•plant$^{-1}$) and (9 kg•plant$^{-1}$) for adhesive and tube grafting methods respectively.

Discussion

In addition to genetic backgrounds and grafting methods, many other factors influence grafting success, including post-grafting environmental conditions, plant vigor, carbohydrate content, and the proper match of vascular bundles (Bisognin et al., 2005). Despite using the same rootstocks, scion and protocol, differences among experiments were noticed in this study (Table 3.1). The explanation for this variation may be due to fluctuation in temperature, light and humidity in greenhouse during the healing process, which may affect the environment on the healing chamber. Fluctuation in the temperature was observed between experiments, where the higher variation was in late autumn and
late spring (7 °C and 4 °C, respectively), while during in spring temperature fluctuation was of (2 °C in average) during the three weeks of the study. Light intensity also fluctuated among experiments where in spring light intensity went from lower to upper gradually as the plants were healing. On the other hand in late autumn was inverted and the fluctuation was highly between days (data not shown). The late spring (2012) the light intensity registered in the greenhouse was lower compared to previous experiment. We attribute this environment variation in the greenhouse to natural fluctuation because for all the experiments, temperature was set at 25/30 °C nigh/day and no additional light was provided.

Since the experiments were carried out during different seasons of the year including late autumn (Exp. 1 and 2), spring (Exp. 3), and late spring (Exp. 4 and 5), the day length could affect the grafting success due to limited or excess light available. In addition to day length, light intensity varies among the seasons. Direct sunlight may cause the healing chamber to heat up excessively or cause the grafted plants to lean towards the light, pulling the graft apart. This natural environmental variation still affects the plant in the healing chamber, specifically the temperature, relative humidity and amount of light absorbed by the shade cloth and plastic of the healing chamber (Johnson and Miles, 2011). Regardless of variance among experiments, survival rate were consistently higher using adhesives and for future work this factors should be, took into account to maximize the grafting success.

Graft healing and survival depends on the compatibility of scion and rootstock combinations, which can be affected by anatomical, physiological, and genetic variables.
Differences in graft success among tomato rootstocks are attributed to incompatibility between rootstocks and the scion used in this study. When tomato cultivars and wild plants (*S. habrochaites*) were used as a rootstock in grafting eggplant, the grafting success was low (Gisbert et al., 2011). The factors that determine how a rootstock and scion interact to produce a strong graft are still not very well understood. However, grafting failure often occurs at early stages of tissue joining (Martinez-Ballesta, 2010), and could be classified into four categories: (1) failed bud growth, (2) virus-caused graft failures, (3) mechanical obstruction at the union and (4) abnormal union structure (Herrero, 1958; Andrews and Marques, 1993). In this study, failure of the scion meristem to grow (category 1) and abnormal union (category 4) were observed during the three weeks of data collection. We have previously observed poor success using H7998 as a rootstock and hybrids derived from this bacterial wilt tolerant parent (unpublished results).

The benefits of adhesive cyanoacrylates or related products have been shown extensively in other applications for joining tissues (Kinloch, 1997; Andrade et al., 2001; Quintino and Pires, 2004; Albert and Job, 2004; Albuquerque et al., 2006; Pawar et al. 2008; Saska et al., 2009 and Singer et al., 2008). Adhesives were used in grafting plants in combination with grafting robots (Oda and Nakajima, 1992; Lee and Oda, 2003). However, performance data has not been published (Kurata, 1994). In this study, we evaluated the benefits of ethyl-2-cyanoacrylate (Krazy Glue) to increase grafting success in tomato seedlings (Figure 3.3). Results revealed that survival was raised for Cherokee Purple grafted onto nine different rootstocks using adhesive. A higher survival rate is
necessary to justify the added cost and labor of grafting and increase the returns for the farm or grafted plant grower.

Cyanoacrylate adhesives increased the rate of success in all rootstocks. Grafting success depends on the rootstock-scion interactions. The perfect combination of the rootstocks and scion parts will result in a successful plant with the potential to impart both abiotic and biotic stress in a given environment without decreasing yield or fruit quality. The improvement of grafting success across all genotypes suggests that adhesives may increase tissue union between rootstock and scion. This improvement could be due to the fast dry of the adhesives, and then the new seedling grafted is firmly held by this production. Thus, by drying fast it leaves minimum or no space between rootstock and scion. The same time alignment can be simplified and more tissues can be matched between the scion and rootstock, which will result in increased grafting success. Yield in open field study was not negatively affected by the use of adhesives in the grafting process. Furthermore, it was detected numerically higher yield compared to tube grafting for both rootstocks evaluated.
### TABLES

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<th>Source of variation</th>
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<td>0.3965</td>
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Nine rootstock genotypes were compared for graft success LA1589 (*S. pimpinellifolium*, an inbred selection from LA2204 (*S. habrochaites*), FG02-188 (*S. lycopersicum*), Hawaii 7998 (H7998); the hybrids SGH07-316, SGH07-315, SGH07-320, SGH07-326; and Maxifort).

*Standard tube graft using a Tygon tubing clip; grafting using adhesive in place of the clip.*

*Experiments were repeated five times, the first and second experiment in late autumn (17 and 24 November 2009 respectively), third experiment in spring (24 March 2010), fourth and fifth late spring (17 and 24 May 2012 respectively).*

*Data were collected at week 1, week 2 and week 3 post-grafting.*

Table 3.1: Analysis of variance for success rate of grafting ‘Cherokee Purple’ scion onto nine different rootstocks using two different grafting methods.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
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<tr>
<td></td>
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<td>Grafting success (%)</td>
<td>Grafting success (%)</td>
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<td>76 a</td>
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<td>SGH07-326</td>
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<td>57 a</td>
<td>42 c</td>
</tr>
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</table>

$^2$Twenty-five plants were grafted per genotype/grafting method and 5 experiments were conducted over time.

$^3$Similar letters in each column indicate nonsignificant differences among rootstocks at $P = 0.05$ based on least significant difference (LSD).

Table 3.2: Effect of rootstock genotype on success rates across all treatments at weekly intervals following the grafting process.
FIGURES

Figure 3.1: Effects of rootstock genotype on grafting success across all five experiments and grafting methods (Adhesives and tube grafting methods).

Y-axis is the percentage of plants that were alive and growing after three weeks post-grafting. In the X-axis nine rootstock genotypes were compared for graft success LA1589 (*S. pimpinellifolium*, an inbred selection from LA2204 (*S. habrochaites*), FG02-188 (*S. lycopersicum*), Hawaii 7998 (H7998); the hybrids SGH07-316, SGH07-315, SGH07-320, SGH07-326; and ‘Maxifort’) using a common scion (Cherokee Purple). Data represent the means and error bars indicate ± SE of five independent experiment (n=125).
Figure 3.2: Main effects of grafting method on grafting survival success across all five experiments and rootstock genotypes.

Y-axis is the percentage of plants that were alive and growing after one, two and three weeks post-grafting. In the X-axis two grafting methods (A = Grafting using adhesives; T = tube grafting) were compared. Data represent the means and error bars indicate ± SE of five independent experiment and nine rootstocks (n=1125).
Figure 3.3: Effects of rootstocks genotype and grafting method on grafting success at week 3 post-grafting.

Data represent the means and error bars indicate ± SE of three independent experiment (n=75). (A = Using adhesives; T = Tube grafting) three experiments (During Spring to late spring).
Chapter 4: Analysis of Quantitative Trait Loci (QTL) 
associated with graft failure in tomato

Abstract

Grafting failure can be caused by multiple factors and can occur at early or late life cycle of the grafted plant. This failure often is attributed to grafting compatibility between scion and rootstock. The ability to predicted failure or success of grafting would help to avoid use of non-compatible rootstock and scion partners. We examined grafting failure and conducted a quantitative trait loci (QTL) analysis to elucidate the genetic basis of grafting failure. We evaluated an inbred backcross population (BC$_3$S$_4$) and an F$_2$ population developed from crosses elite lines of *Solanum lycopersicum* L. (H7998 × OH88119) and (H7998 × Ohio MR13), respectively. H7998 had been identified as a parent with poor grafting success. We collected survival data for 3 weeks post-grafting. Scion height was also recorded for the F$_2$ population at week 3 post-grafting. We used single factor analysis of variance to detect marker-trait association. Our results suggest that a putative QTL is underlying grafting failure on chromosomes nine, and two QTLs for scion growth (height) on chromosome 2 and 4. If confirmed, these results can be exploited for rootstock improvement and to gain further understanding of QTL underlying graft failure during the healing process.
Introduction

Grafting is the ability to unify two plants, the scion (upper plant) and the rootstock (bottom plant) in order to produce a single plant (Kramer, 1979; Andrews and Marquez, 1993). This technique has historically been used in fruit trees due to their extended reproductive lifecycle (Kramer, 1979). During the early 1920’s this technique was introduced into annual crops to reduce diseases caused by soil pathogens (Lee, 1993). Later, it was used to impart abiotic tolerance and to enhance nutrient uptake in vegetables (Lee et al., 1998; Kubota et al., 2008; Rivard et al., 2010).

The success of grafting depends on various factors including taxonomic differences, environment, availability of oxygen and water, physiological stage of rootstock/scion, diseases, pests, herbicide toxicity and the ability of the grafter to perform successful grafts (Andrews and Marquez, 1993). A success in grafting encompasses a complex biochemical and structural sequence of events: (1) wound response, from rootstock and scion; (2) callus bridge formation; (3) vascular differentiation; and (4) production of secondary xylem and phloem. Extensive reviews detailing these steps have been published elsewhere (McCully, 1983; Moore, 1984; Andrew and Marquez, 1993; Kawaguchi et al., 2008; Martinez-Ballesta et al., 2010). The success of grafting in plants depends on the successful development of the events noted above between the scion and rootstock.

Grafting is not always successful; multiple factors contributed to grafting failure (Masa, 1989; Leoni et al., 1990; Edelstein, 1999, 2004). Poor development of the graft union due to anatomical mismatching, poor craftsmanship, adverse environmental
conditions, disease, and graft incompatibility (Pease, 1933; Herrero, 1951; Andrews and Marques, 1993; Hartmann et al., 2002). Graft incompatibility is defined as the interruption or delay in the development of the new grafted seedling or growing plant (Santamour and Frank, 1988; Kollman and Glockman, 1985). This interruption can have an anatomical basis (Mosse, 1962), be affected by physiological intolerance at the cellular level (Feucht, 1987; Moore and Walker, 1981), and be caused by virus or phytoplasma transmission (Mircetich et al., 1980; Uyemoto, 1989; Hartman et al., 2002). The examples of virus-induced incompatibility include black line in English walnut caused by cherry leaf roll virus (Mircetich et al., 1980), prune brown line caused by a strain of tomato ring spot virus (99; Mircetich and Hoy, 1981) and apple union necrosis and decline caused by virus (Cummins and Gonsalves, 1982). Incompatibility could also be directly related to undergrowth and overgrowth of the scion relative to that of the rootstock, due to unknown reasons (Salesses and Al Kai, 1985; Lee, 2006).

In tomatoes, a limitation to grafting success is proposed to be Tobacco Mosaic Virus (TMV) and resistance genes. Three different alleles (Tm-1, Tm-2 and Tm-2a) against TMV have been identified. Plants possessing the Tm-1 allele are symptomless or tolerant to TMV, those possessing the Tm-2a allele confer a hypersensitive response, and those with the Tm-2 allele are intermediate (Hall, 1980; Yamakawa, 1982; Oda, 1995; King et al., 2010). In 1981, Yamakawa suggested that different combinations of these alleles in tomato rootstocks and scions could influence whether the grafting process is successful or not. To our knowledge, this is the closest genetic study that has been conducted for grafting compatibility due allelic interaction between scion and rootstock.
Most studies of grafting failure have focused on the anatomical, physiological and biochemical factors (Moore, 1983; Kawaguchi et al. 2008 and Andrew and Marquez, 1993). Association between genetic relationships and graft failure was suggested for grafts peach-plum (Grasselly, 1968), almond-plum (Kester et al., 1965) and Copes (1973), studied the inheritance of graft compatibility in Douglas-fir, suggesting multiple genes are involved in this process. On the other hand, studies in human, frogs and mice demonstrated the relationship between genetics and graft incompatibility (Ceppellini et al., 1966; Bistoni et al., 2012). Despite of numerous studies conducted on grafting success in plants, the information of genetic basis of grafting failure was not revealed. The objective of this study was to identify the genetic basis of grafting failure between tomato rootstocks and scions during the early portion of the lifecycle.

**Materials and Methods**

*Plant materials and experimental design*

Two segregating populations were used to determine the genetic basis of graft failure using marker-trait analysis. Our experience with H7998 suggested that this parent had a low rate of success during grafting with multiple scions (e.g. Celebrity, NC84173, Cherokee Purple and Ohio FG99-218). The initial population was created by crossing the H7998 (low grafting success) × OH88119 (high grafting success). An inbred backcross (BC3S4) population was developed using OH88119 as a recurrent parent details of this population are found somewhere else (Yang et al., 2005). The population size for this study consisted of 172 BC3S4 families. The second population consist of 288 F2 plants
from a cross between H7998 (low grafting success) and Ohio MR13 (high grafting success). A common scion was used in both experiments a fresh marker material “NC84173”.

All tomato seeds were sown in 288-cell flats with a volume of 13 mL (Hummert, EARTH City, MO-USA) using PRO-MIX (Premier horticulture, Quakertown, PA-USA). A month after seeding, the rootstock seedlings were transplanted into 40-cell trays with a cell volume of 68 mL (Hummert, EARTH City, MO-USA) using PRO-MIX. The seedlings were grown for one additional week prior the grafting process in the greenhouse. Temperatures were set at 24/18 °C day/night and a 12 h of photoperiod.

Two independent experiments were conducted in the greenhouse facilities at the Ohio Agricultural Research and Development Center (OARDC) in Wooster, Ohio. The initial experiment was a randomized complete block design (RCBD) with two blocks. The BC$_3$S$_4$ families (OH88119 × H7998) were used as rootstocks and NC84173 was used as the scion. Ten plants were grafted per each family. In the second experiment, F$_2$ plants derived from OhioMR13 × H7998 were used as rootstocks and NC84173 was used as the scion. A completely randomized design (CRD) was used distributing 288 F$_2$ plants in the healing chamber after the grafting process.

**DNA extraction**

Leaf tissue of approximately 1-cm$^2$ was collected from each rootstock used in the grafting process for the F2 population. The leaf tissue for BC3S4 was bulked from the 10 plants used as a rootstock for each family. Total genomic DNA was extracted using the
modified CTAB protocol (Kabelka et al., 2002) scaled for 96-well format. Polymerase chain reaction (PCR) was conducted as described previously (Yang et al., 2005; Robbins et al., 2010; and Wang et al., 2011).

**Molecular markers and genotyping**

The initial BC$_3$S$_4$ population was genotyped using 78 polymorphic markers including, simple repeat (SSR), Insertion/deletion (INDEL), and Single Nucleotide Polymorphism (SNP) were selected based on their ability to distinguish H7998 and OH88119 (Yang et al., 2005). These markers were distributed across the genome with 2-17 markers per chromosome. The F$_2$ population was genotyped using 40 Single Nucleotide Polymorphism (SNP) markers as implemented in the Infinium Golden-gate assay (Illumina Inc., San Diego, CA, USA) to detect polymorphisms between H7998 and OhioMR13. The forty SNP markers were scattered throughout the genome.

Genotyping was conducted on three platforms, one for size polymorphism (SSR and INDELS). Size polymorphisms were detected using polyacrylamide gels on the Li-Cor-IR2 4200 system (LI-COR biosciences, Lincoln, NE) or agarose gels. SNPs were detected by an Allele Specific Primer Extension (ASPE) assay (Lee et al., 2004). Samples were loaded at 50 ng/ul on an Ilumina BeadXpress analyzer system (Luminex Corporation, Austin, TX). For the second experiment, Golden-Gate assay was performed following manufacture’s protocol of Ilumina (Ilumina, San Diego, CA).
**Grafting and healing process**

Seven days following transplanting, rootstocks were grafted using cleft grafting method (Oda, 1999). The stem of the scion were cut in a wedge, and the tapered scion was fit into a cleft cut in the end of the rootstocks. The graft union was held firm with a plastic tube to ensure contact vascular tissue between partners (scion-rootstock) (Oda, 1999).

The grafted plants were placed in a 5.5 m × 1.2 m × 0.7 m dimension healing chamber constructed from ¾ - inch PVC pipe as a frame and 0.08-mm plastic covering to maintain high humidity. The greenhouse bench was covered with capillary mat (Agriculture solutions, Columbus, IN) which was maintained saturated with water. Shade cloth (60% shade) was used for the first week to minimize water loss through transpiration. Temperatures were maintained at 28-30°C. After 7 days, the shade cloth was gradually removed and the healing chamber was opened to increase light and lower humidity. By 14 days post-grafting, the shade cloth and plastic were completely removed.

**Phenotype for grafting success**

Grafting failure was measured as percent survival of the 10 rootstocks grafted per each BC₃S₄ family at week-3 post-grafting. For the F₂ population, survival was determined on each individual rootstock; data was collected at week 1, week 2 and week 3. We also collected the height of the scion grafted during the final week (week 3). An independent experiment was carried out during springtime of 2013. We grafted 120 plants in combinations: H7998 self-grafted (autograft), H7998 with NC84173.
(homograft) and NC84173 self-grafted (autograft). The experimental design was a randomized complete block design with four blocks. Survival data was collected along the three weeks post grafting.

**Statistical analysis**

Best Linear Unbiased Predictors (BLUPs) were estimated in order to account for the variance associated with different grafters and experimental replications for BC3S4 population (H7998 × OH88119). The model used estimation of BLUPs was:

\[
Y_{ijk} = \mu + R_i + G_j + E_k + \text{RE}_{ik} + \text{RGE}_{ijk} + \varepsilon_{ijklm}
\]

where \( Y_{ijk} \) was the percentage of grafting success, \( \mu \) was the overall mean, \( R_i \) was the effect due to ith rootstock genotype, \( G_j \) was the effect due to grafter, \( E_k \) was the effect due to kth experiment. Two and three way interactions were added to the model including, \( \text{RE}_{ik} \) was the effect due to the interaction between rootstock genotype and experiment, \( \text{RGE}_{ijk} \) was the effect due to the three-way interaction between the tree main effects, and \( \varepsilon_{ijklm} \) was the experimental error. In this design, all effects were random.

For the estimation of BLUPs Lme4 package (Bates et al., 2011) was used in “Rstudio” computer software version 0.97.320 (RStudio, 2012) in concert with the R core package version 2.15.3 (R Core team, 2012).

To determine the association between grafting success and DNA-based markers, data were analyzed using analysis of variance (ANOVA) based on general linear model. For statistical analysis, we used the statistical package in “Rstudio” computer software
The model used was:

\[ Y_{ij} = \mu + M_i + \varepsilon_{ij} \]

where \( Y_{ij} \) was the BLUPs value for grafting success (phenotype data), \( \mu \) was the overall mean, \( M_i \) was the effect due to marker class, and \( \varepsilon_{ij} \) was the experimental error. The approximate F test for marker-trait associations (\( M_i \)) was \( M_i/\varepsilon_{ij} \).

Marker-trait associations were tested in the F2 population using analysis of variance (ANOVA) through the general linear model. Phenotypic data was represented by the mean values of survival (dead = 0, stunted = 1, alive = 2) of the grafted seedling during three weeks post-grafting. For the last week (week 3), the phenotypic value was represented also by the mean values height scion. The model used was:

\[ Y_{ij} = \mu + M_i + \varepsilon_{ij} \]

where \( Y_{ij} \) was the quality measured (survival values and high of scion), \( \mu \) was the overall mean, \( M_i \) was the effect due to marker class, and \( \varepsilon_{ij} \) was the experimental error. The statistical package in RStudio computer software version 0.97.320 (RStudio, 2012) was used in concert with the R core package version 2.15.3 (R Core team, 2012).

For the independent experiment, ANOVA was performed to determine grafting capacity of H7998. The Agricolae package in R (De Mendiburu, 2012) was used to perform mean separation. Mean separations were based on Tukey’s test \( (P < 0.05) \).
Results

The marker data was obtained from genotyping the populations BC$_3$S$_4$ with a total of 78 markers. Markers were distributed from 2 to 19 markers per chromosome. In F$_2$, population markers were mapped from 0 to 7 markers per chromosome, with a total of 40 markers. Our aim was to identify at least one marker per chromosome; however the marker coverage was uneven with only two markers on chromosomes 8 and 12 in BC$_3$S$_4$ population and no marker was present on chromosome 5 in F$_2$ population.

The frequency distribution of the grafting success/failure effect in the BC$_3$S$_4$ families were continuous (Figure 4.1-A), indicating that grafting success/failure is controlled by multiple genes. The BLUPs-value ranged from -100 to 130 (Figure 4.1-A). The survival data for F$_2$ population show binary distribution (Figure 4.1-B); it shows a clear segregating population. The scion height phenotype for F$_2$ population show skewed distribution (Figure 4.1-C). The skewed distribution appears to be due to the high number of plants that died before week 3, which is, continuous and not binary.

Analysis of marker-trait associations demonstrated potential linkage between markers and grafting failure on chromosome 9 and chromosome 5. On chromosome 9, a marker SSR70 is highly associated with percentage of graft failure ($P = 0.002196$). Alleles from the H7998 parent are associated with grafting failure (BLUPs value = -28.69). There is some evidence for a negative association with OH88119 alleles on chromosome 5 (Table 4.3).

F$_2$ population (H7998 × Ohio MR13) was developed to confirm the genetic basis of grafting failure in allele coming from H7998. To facilitate the analysis of marker-trait
association, we decided to collect one objective phenotype data (height of scion) and survival data, at final week (week 3). Using these data, we detected two markers on chromosome 2 and 4 that were significantly associated with low scion height (Table 4.4; Table 4.5).

The survival data for F$_2$ population did not follow 1:3 segregation ratios, which indicates that multiple genes determine grafting failure. This result agrees with the BC$_3$S$_4$ phenotype data, since it revealed continuous distribution of the BLUPs-value. In F$_2$ population, the markers sol_cap_snp_sl_20325 and sol_cap_snp_sl_8464 on chromosome 2 were highly associated with grafting failure. The rank of means indicates alleles associated with Ohio MR13 have higher grafting survival compared to H7998, and the heterozygous resulted in the survival mean (Table 4.3). This result is inconsistent with the allelic effect observed in the BC$_3$S$_4$ population, where H7998 genotypes had the lowest graft compatibility with the scion NC84173.

The growth of scion varied markedly among the F$_2$ rootstock population. Association was detected on chromosome 2, 4 for growth of scion. On chromosome 2, a marker Sol_cap_snp_ls_20052 was highly associated with scion growth ($P = 0.04600$). Alleles from H7998 parent is associated with reduction of scion growth, the mean height 3.59 cm compared to 7.65 cm for plants associated with alleles from Ohio MR 13. On chromosome 4 we observed the same trend with marginal significance on association between low scion height and alleles from H7998 parent (Table 4.5).

After a period of 3 weeks, the grafting failure expressed in success value for H7998-self-grafted decreased from 1.1 to 0.3, H7998 × NC84173 1.4 to 0.8, and
NC84173 self-grafted 1.7 to 1.5 ($P < 0.001$). This result strongly suggests that H7998 presents grafting rejection not only foreign tissue also to its own (i.e. intolerance to grafting) (Table 4.6). This phenomenon was before reported in Douglas-fir (Copes, 1974).

**Discussion**

The goal of this research was to assess the genetic basis of grafting failure in populations derived from H7998 × OH88119 and H7998 × Ohio MR13 via single factor QTL analysis.

We used grafting success and scion height for evaluation of failure at an early stage in tomato. Two populations were used as rootstock and one common scion in two independent experiments. Grafting success was recorded along the 3 weeks-post grafting. In the BC$_3$S$_4$ population (H7998 × OH88119), survival of 10 plants was used as percentage of survival, while in F$_2$ population (H7998 × Ohio MR13), the data collected data was categorical including; dead = 0, stunted = 1 and alive =2. We identified a potential marker from H7998 that was associated with the grafting failure. Single-marker analysis in BC$_3$S$_4$ population implicates one genomic location chromosome 9. The phenotype data suggest that this trait is a quantitative trait. This result supports the early suggestion that grafting compatibility is a quantitative trait that is controlled polygenically (Copes, 1973).

Subsequent testing in F$_2$ population, we fail to validate the marker on chromosome 9 associated with grafting failure with allele from H7998 parent.
Nevertheless, the phenotype data (scion height) show a continuous distribution, confirming that grafting failure is a trait controlled by multiple genes. Even we fail to validate the marker on chromosome 9 we found association of grafting failure on chromosome 2 associated to allele of H7998 parent. These results prove the importance of confirming the findings when associating traits of interest.

Our results from two independent populations have not validated the markers associated with grafting failure unanimously. QTL effect size, high environment influence, lack of overlap marker set between populations limited our ability to identify the QTL associated with grafting failure. Quantitative trait loci (QTL) with small effects on phenotypic variation can be difficult to detect and analyze.

A high level of failure was observed when H7998 was used as a rootstock even when it was auto-grafted. This observation may explain why this specific accession performs poorly when used as a rootstock. Grafting rejection was before reported in Douglas-Fir (Copes, 1974) who suggested it was caused by genetic additive, thus, grafting for compatible rootstock would be possible by genetic selection.
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$^y$RE, restriction enzyme; $^z$ Tem. Annealing temperature of the marker.

Table 4.1: Polymorphic molecular markers used in the population derived from the cross OH88119 × H7998
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\(^3\)RE, restriction enzyme; \(^2\) tem. Annealing temperature of the marker.

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$^3$RE, restriction enzyme; $^2$ tem. Annealing temperature of the marker.
Table 4.1 continued

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<th>ReversePrimer</th>
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$^y$RE, restriction enzyme; $^z$ tem. Annealing temperature of the marker.
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<th>Pos_(bp)</th>
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**Table 4.2:** Single Nucleotide Polymorphisms (SNP) molecular markers used in the population derived from the cross Ohio MR13 × H7998.
Entries with the same grouping letter are not significantly different based on the LSD (0.05).

H7998 = Homozygous for the Hawaii 7998 allele, OH88119 = Homozygous for the OH88119 allele; H = heterozygote.

Table 4.3: Single factor analysis for association in BC₃S₄ (OH88119 × H7998) population for grafting success at week 3.
Survival at week 1

<table>
<thead>
<tr>
<th>Marker</th>
<th>Chromosome</th>
<th>Allele Genotype</th>
<th>Survival Mean</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
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<td>1.71 ab</td>
<td>0.04866</td>
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<td>OhioMR13</td>
<td>1.89 a</td>
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<tr>
<td>sol_cap_snp_sl_20052</td>
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<td>H</td>
<td>1.59 b</td>
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</tr>
<tr>
<td>sol_cap_snp_sl_20052</td>
<td>2</td>
<td>H7998</td>
<td>1.71 ab</td>
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</tr>
<tr>
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<td>OhioMR13</td>
<td>1.94 a</td>
<td>0.07576</td>
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<tr>
<td>sol_cap_snp_sl_20052</td>
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<td>H</td>
<td>1.63 b</td>
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</tr>
<tr>
<td>sol_cap_snp_sl_8000</td>
<td>10</td>
<td>H7998</td>
<td>1.60 a</td>
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<tr>
<td>sol_cap_snp_sl_8000</td>
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<td>OhioMR13</td>
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<td>0.08621</td>
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<tr>
<td>sol_cap_snp_sl_8000</td>
<td>10</td>
<td>H</td>
<td>1.83 a</td>
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Survival at week 2

<table>
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<th>Allele Genotype</th>
<th>Survival Mean</th>
<th>P-value</th>
</tr>
</thead>
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<td>sol_cap_snp_sl_8464</td>
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<td>OhioMR13</td>
<td>1.39 ab</td>
<td>0.02691</td>
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<tr>
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<td>H</td>
<td>0.91 b</td>
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</tr>
<tr>
<td>sol_cap_snp_sl_20325</td>
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<td>H7998</td>
<td>1.43 a</td>
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<tr>
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<tr>
<td>sol_cap_snp_sl_20325</td>
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<td>0.08454</td>
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<tr>
<td>sol_cap_snp_sl_20052</td>
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Survival at week 3

<table>
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<th>Allele Genotype</th>
<th>Survival Mean</th>
<th>P-value</th>
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</table>

*Data were recorded as (Alive = 2; Stunted =1; Dead = 0)
*Entries with the same grouping letter are not significantly different based on the LSD (0.05).
*H7998 = Homozygous for the Hawaii 7998 allele, OhioMR13 = Homozygous for the Ohio MR13 allele; H= heterozygote.

Table 4.4: Single factor analysis of association with F2 population for grafting success at week 1, week 2 and week 3 post-grafting.
<table>
<thead>
<tr>
<th>Marker</th>
<th>Chromosome</th>
<th>Allele Genotype</th>
<th>Height Mean (cm)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
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</tr>
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<td>sol_cap_snp_sl_20052</td>
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<td>5.09 ab</td>
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<tr>
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<td>4</td>
<td>OhioMR13</td>
<td>6.06 ab</td>
<td></td>
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<td>4</td>
<td>H</td>
<td>6.18 a</td>
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</tr>
<tr>
<td>sol_cap_snp_sl_31883</td>
<td>9</td>
<td>H</td>
<td>6.06 a</td>
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</tbody>
</table>

1Scion height was measured from the graft union to the meristem.
2Entries with the same grouping letter are not significantly different based on the Tukey’s test (0.05).
3H7998 = Homozygous for the Hawaii 7998 allele, OhioMR13 = Homozygous for the Ohio MR13 allele; H = heterozygote.

Table 4.5: Single factor analysis of association with F2 population height of the scion at week 3 post-grafting.
<table>
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<th>Week</th>
<th>Survival Mean$^y$</th>
<th>P-value</th>
</tr>
</thead>
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<td>1.1 b$^x$</td>
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</tr>
<tr>
<td>H7998xNC84173</td>
<td>1</td>
<td>1.4 ab</td>
<td>***</td>
</tr>
<tr>
<td>NC84173 (autograft)</td>
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<td>1.7 a</td>
<td></td>
</tr>
<tr>
<td>H7998 (autograft)</td>
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<td>0.3 c</td>
<td></td>
</tr>
<tr>
<td>H7998xNC84173</td>
<td>2</td>
<td>1.1 b</td>
<td>***</td>
</tr>
<tr>
<td>NC84173 (autograft)</td>
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<td>1.6 a</td>
<td></td>
</tr>
<tr>
<td>H7998 (autograft)</td>
<td>3</td>
<td>0.3 c</td>
<td></td>
</tr>
<tr>
<td>H7998xNC84173</td>
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<td>0.8 b</td>
<td>***</td>
</tr>
<tr>
<td>NC84173 (autograft)</td>
<td>3</td>
<td>1.5 a</td>
<td></td>
</tr>
</tbody>
</table>

***, significant at < 0.001 level

$^x$Data were recorded as (Alive = 2; Stunted =1; Dead = 0)

$^y$Values with different letters were significantly different (P < 0.05)

$^z$Three grafting combinations were conducted H7998 self-grafted, H7998 grafted with NC84173, and NC74173 self-grafted.

Table 4.6: Analysis of variance for survival mean along three weeks-post-grafting in three different grafting combinations.
Figure 4.1: Phenotypic data distribution for grafting success at week-3 post grafting for BC$_3$S$_4$ and F$_2$ Population.

A) BC$_3$S$_4$ population, 172 families Hawaii7998 x OH89119 (BLUPs-value). Population mean = -0.9111, B) F$_2$ population 288 individual plants Hawaii7998 x Ohio MR13 (0 = dead, 1 = stunted, 2 = alive) Population mean = 1.16, C) F$_2$ population 288 individual plants Hawaii7998 x Ohio MR13 scion height (cm). Population mean = 6.117 cm.
Chapter 5: Conclusions

These studies were conducted to expand our understanding of grafting technology and to identify key selection points in a breeding program. Improvements on grafting include increased seed quality when using wild species for tomato rootstocks and increased grafting success using a variety of rootstocks. Grafting offers multiple benefits including disease resistance and tolerance to abiotic stress by using genetic diversity.

Rootstock with multiple resistance and tolerance to biotic and abiotic stresses are required in order to justify the extra cost added in the production. At the same time it is important to obtain high rootstock seed quality. Screening multiple inbred lines crossed with multiple wild relatives can help to achieve these goals. The production of seeds is a complex interaction of genetics and environmental factors.

The grafting process itself also has genetic and environmental components affecting its efficiency, which are measured as grafting success/failure. Increasing its efficiency by implementing new methods could make grafting available to more farmers. Another approach is to create a rootstock that is highly compatible with multiple varieties (scion). Finding the genetic basis of grafting failure may allow for breeding rootstock with multiple resistance genes and high success rates.
The first objective of this study was to optimize screening and early selection of new potential crosses that produce high seed yield and quality. Each cross was evaluated in terms of seed production (fruit set and seed yield) and seed quality (seed size and germination). The greatest source of variation for fruit set was found among environments (experiments) which accounted for 22% of the total phenotypic variation. In contrast, for seed yield the genetic variation and environment was similar 28% and 27%, respectively. The source of variation for seed weight was mainly genetic components, which accounted for 84% of the phenotypic variation. Finally, seed germination was influenced by genetic components 65% and environment 13% of the phenotypic variation. We thus suggest to increase seed production environment must be optimize, while increase seed quality genetic selection must be performed before the crosses are conducted.

In addition, the effect of fruit maturation on seed quality was determined from eight different crosses harvested at three different times (Breaker, full ripe and overripe). The seeds harvested from the overripe fruits resulted to have high final seed germination and the speed was higher. However, we identified a female parent FG02-188 that produces high seed quality regardless of the male parent used and harvesting time. Fruit maturation can be used as a tool to increase seed quality in hybrid seed production, also can be used to select some hybrids that have high seed germination regardless of fruit harvesting.

Early selection is important in order to save resource and reduce cost in production system. Seed size was used as a predictor for seed germination with the
objective of to identify a selection point based on this trait. The selection point was based on the minimum national and international standard seed germination 75% (AASCO, 2013) and 85% (ISF, 2013). We identified three selection points with different percentage of discharging bad seed quality and retaining high seed quality for seed germination test. Selection at 1.4 mg seed weight, 100% of hybrids with high seed quality can be saved the same time 33% of the hybrids with low seed quality can be omitted. The selection point can be increase to 1.8 mg where 74% of the hybrid with high seed quality can be retained and omitted 43% of hybrids with low seeds quality. Finally, at 2.2 mg it can be retained only 53% of the hybrids high seed quality and omitted 62% of the hybrids with low seed quality. These results can be used to draw thresholds depending on resources available for further seed testing (germination).

The second objective was to increase efficiency of grafting success using adhesives in the grafting process. Nine different rootstocks were used grafted with two different methods tube grafting and using adhesives. The experiments were repeated in different seasons under greenhouse environments. Grafting success was measure a survival plant at week 3 post-grafting. Additionally, one season of field experiment was conducted to evaluate the effect of grafting methods on open field conditions. The analysis of variance (ANOVA) showed that grafting method had a significant effect of grafting success and we identified high environmental effect and rootstock genetics. Thus, adhesives can be a tool to improve grafting success in tomato or other vegetables.

The third objective was to identify the genetic basis of grafting failure between scion-rootstocks via quantitative trait loci (QTL) analysis. The population consisted of
advanced backcross BC$_3$S$_4$ population derived from OH88119 and H7998, and F$_2$ population derived from Ohio MR 13 and H7998. The initial experiment phenotype data was collected as a percentage of survival for 10 plants from each BC$_3$S$_4$ family. In the second F$_2$ population data was collected as dead = 0, stunted = 1, alive=2, and height of scion was recorded at week-3 post grafting. QTL analysis was based on single factor analysis of variance. The initial findings suggest on BC$_3$S$_4$ population one QTL was identified for grafting failure. In the second F$_2$ population, a new QTL was identified on chromosome 2 that is associated with low height scion. Additionally, we determined that H7998 is intolerant to the grafting process, which would explain the poor grafting success in previous studies. However, it is unknown the cause of poor performing of H7998 to the grafting process, it may be genetically limited to tolerate high humidity, unable to overcome the stress caused by grafting (poor wound response) or other causes.

Combining fruit setting ability, high seed yield, seed size and seed germination, will be possible to increase seed yield and quality for new hybrid tomato seeds for rootstocks. We were able to identify potential rootstocks with high seed quality and high seed production. In general, grafting efficiency can be improved using adhesives as tool to bind the grafted seedling in the process. The results of this study show that grafting failure in tomato is associated with alleles from the H7998 parent. We also identified that grafting rejection happens in H7998 even when is self-grafted. More research is needed to identify a marker for the alleles that causes the grafting failure in H7998 or other causes. Finding markers or genes that causes the grafting rejection would help to
accelerate the breeding of highly compatible rootstocks with many scions, which is the most desirable trait for a farmer or grafter producer.
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