Project title:	Combining root architecture, root function and soil management to improve production efficiency and quality of apples
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Project leader:	Richard J. Harrison, NIAB. Felicidad Fernández Fernández, NIAB EMR. Amanda Rasmussen, University of Nottingham. Nicola Harrison, AHDB.
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Key staff:	Magdalena Cobo Medina, NIAB EMR.
Location of project:	NIAB EMR
Industry Representative:	NA
Date project commenced:	1st October 2017

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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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[Organisation]

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[Name]

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[Organisation]

Signature ..... Date .....

Report authorised by:

[Name]

[Position]

[Organisation]

Signature ..... Date .....

[Name]

[Position]

[Organisation]

Signature ..... Date .....

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GROWER SUMMARY

Headline

The next step in identifying the genes for rootstock dwarfing has been achieved. The chromosomal regions containing the dwarfing genes have been narrowed down from 4.4Mb to 2.1Mb in chromosome 5 and from 5.8Mb to 1.8Mb in chromosome 11.

Background

Most of the temperate tree fruit crops grown in commercial production are grafted onto rootstocks selected for specific traits such as growth control, early fruit production, precocity, pest and disease resistance, soil anchorage and cold hardiness. Rootstocks are an essential component of successful tree fruit production, conferring productivity through altering the floral and vegetative balance. Growth-controlling rootstocks produce compact trees and have facilitated the transition of low-yielding traditional orchards to high-density modern orchards. However, breeding dwarfing rootstocks is difficult since the dwarfing effect is usually lost over generations, therefore new molecular markers strongly linked to rootstock-induced dwarfing are essential to hasten rootstock breeding.

Root bark is described as all tissues outside the cambium layer and a high proportion of root bark in the root of an apple rootstock has been associated with rootstock-induced dwarfing. A Quantitative Trait Loci (QTL) map for root bark ratio identified three regions in the genome controlling the root bark. Two of these regions colocalise with areas previously linked with dwarfing although the genes controlling this complex mechanism have not yet been discovered.

Recent studies at NIAB EMR have identified different types of root system architecture (RSA) associated with commercial rootstocks utilised in apple orchards, indicating a relationship between dwarfing and RSA. However, RSA is poorly understood, despite the importance of RSA for optimising both productivity and resilience, as global challenges drive the need for more efficient and resilient crop production.

The overall aim of this project is to assess the effect of dwarfing on RSA and ultimately to assess the impact of dwarfing-associated roots on nitrogen uptake efficiency.

This work will generate molecular markers for dwarfing and root traits which will assist the rootstock breeding programmes. Furthermore, understanding the movement of nitrogen in apple rootstock will allow us to advise growers on sensible fertilization protocols. Additionally, this research could have a significant impact for other high value perennial crops including pear, cherry and apricot.

Summary

In the third year of this project, root bark development data revealed that all the rootstocks have a high root bark percentage at an early stage of development and this is decreasing as they develop. At the end of the first growing season, differences in root bark percentage between dwarfing and vigorous rootstocks start to appear but these differences are not fully expressed. All this information suggests that during the first growing season there are fundamental differentiation processes occurring which will lead to differences in root bark proportion. Therefore, more studies should be focused on this time frame to better understand these mechanisms.

The dwarfing markers previously developed were screened on rootstocks available in the breeding trials at NIAB EMR. New recombinants were detected and root segments were collected from all the trees at the end of the trials. Recombinants identified from these breeding trials allowed us to narrow down the genetic regions containing the dwarfing genes. The region located in chromosome 5 was narrowed down from 4.4Mb to 2.1Mb and the region located in chromosome 11 from 5.8Mb to 1.8Mb. Consequently, the number of genes contained in the dwarfing regions has dramatically decreased and this will facilitate the identification of potential genes controlling dwarfing. Currently, the rootstock breeding programme at NIAB EMR is using the markers developed in this project to help them with the rootstocks breeding selections. In addition, this dataset showed that the scion also has an impact on root bark percentage. Further investigations will be conducted to understand this intriguing interaction.

An experiment to assess the impact of dwarfing on root system architecture has been performed during 2020 using a mapping population from a cross of Golden Delicious and M9, which segregates for vigour. This will allow us to map important root traits such as rooting ability, root thickness and total root area and markers for these traits could be created.

Financial Benefits

The financial benefits cannot be quantified at present. However, this work will provide fundamental insights into the genetic basis of root system architecture in apple rootstocks, generating molecular markers for dwarfing and root traits. These markers can then be deployed into breeding programmes to aid the generation of new and improved rootstocks to benefit the industry.

Action Points

There are no grower action points at this early stage of the project.

SCIENCE SECTION

Introduction

Malus domestica

Apples have been cultivated since ancient times and are among the most economically important fruit crops with more than 7500 known cultivars and twenty-five reported species of *Malus* (Noiton & Alspach., 1996; Dobrzañski et al., 2006). This crop is primarily grown in temperate areas due to the chilling requirements for the initiation of blossoming (Heide and Prestrud 2005).

Over 5 million hectares are grown worldwide, with more than 80 million tonnes of fruits produced in 2017 (FAOSTAT 2017). In the past 30 years, there has been a global increase of 117% in apple production, which is mainly attributed to a higher productivity per hectare since the area utilised for apple production has only increased by 20% during the same period (FAOSTAT 2017).

The cultivated apple, *Malus domestica* Borkh, belongs to the Rosaceae family, and the subfamily Maloideae**,** which also includes other tree-fruit species like pears, quince, loquat and medlar (Evans et al., 2002).

Apple rootstocks

Rootstocks are defined as the part of the tree containing the root system and have been used in temperate fruit trees for more than 2000 years (Webster, 1995). At the beginning of the twentieth century, apple rootstocks from around the world were collected, classified into nine types from I to IX and described at the East Malling Research Station (UK) (Hatton, 1917).

Apple rootstock 'M9' (Jaune de Metz), was released in 1914 at East Malling Research Station in Kent, (UK) (Webster, 1995). Since then, it has become the most widely used dwarfing rootstock despite also having some drawbacks including poor soil anchorage and fireblight (*Erwinia amylovora*) susceptibility (Ferree et al., 1993; Norelli et al., 2003). A large number of apple rootstock varieties used worldwide have parentage derived from M9 like "M27", "P22" and "Ottawa 3" (Cummins et al., 1983).

A few decades later, a new series of rootstocks was produced in collaboration with the John Innes Centre, then located in Merton (UK). These were named as Malling Merton series (Preston., 1955). Among these rootstocks, it is worth highlighting MM106 and MM111 which are commonly used in modern day apple orchards (Webster et al., 2000) (Figure 1).

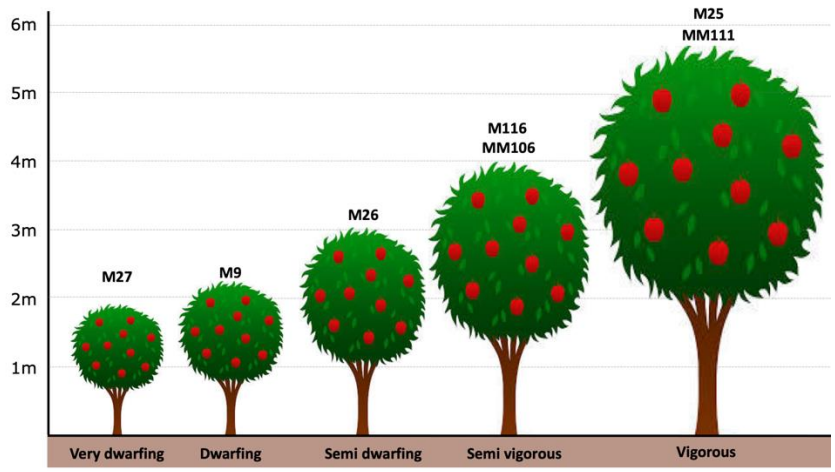

Figure 1. Malling apple rootstocks and their influence on tree size

Rootstocks have always been selected for a wide range of desirable characteristics such as pest and disease resistance, cold hardiness, good soil anchorage, reduced suckering as well as precocity and tree size (Cummins et al., 1983; Pilcher et al., 2008). Furthermore, the root system of the tree plays a crucial role in nutrient uptake and adaptation to water deficit (Marguerit et al., 2012). For all of these reasons, the choice of an appropriate rootstock is fundamental to orchard success. With the increasing global demand for food, rootstock selection is now gaining more importance since rootstocks can contribute to the adaptation of water deficit and resistance to plant pathogens and therefore, would have an effect in yield (Jensen et al., 2012; Marguerit et al., 2012; Tamura, 2012).

Rootstock induced dwarfing

Rootstock-induced dwarfing is a complex trait which is affected by several factors such as environmental conditions, growth parameters and scion variety (Foster et al., 2015). Many hypotheses have been proposed to explain dwarfing, most of them related to the altered root-to-shoot and shoot-to-root chemical signalling (Gregory et al., 2013).

Dwarfing rootstocks are especially important since they reduce the size of the grafted scion and also induce a higher proportion of buds to flower (Atkinson, 2001; Seleznyova et al., 2003).

For these reasons, dwarfing rootstocks are essential to intensive production methods, since they contribute to a greater yield per unit area and are also capable of cropping earlier (Robinson, 2007).

Previous studies demonstrated that a high proportion of root bark percentage in the root of an apple rootstock is associated with rootstock induced dwarfing (Beakbane and Thompson, 1947). It also showed that vigorous rootstocks have more vessels and xylem fibres than the dwarfing rootstocks. However, dwarfing rootstocks have two to three times as much living tissue as dead tissue compared with vigorous rootstocks (Beakbane, 1940 & 1947).

Several studies have identified QTLs associated with rootstock-induced dwarfing on linkage groups 5, 11 and 13 (Pilcher et al., 2008; Fazio et al., 2014; Foster et al., 2015; Harrison et al., 2016b)**.** A QTL map for root bark ratio, a primary trait related to dwarfing, was performed using the progeny of a cross of M27xM116 (M432 population). Two QTLs for root bark percentage, named RB1 and RB2, were found to co-localize to regions previously identified as controlling dwarfing in chromosomes 5 and 11 and a new region in linkage group 13 was also identified (Harrison et al., 2016b).

QTLs for early bearing, rootstock height, tree height, fruit count and flower density roughly co-localise with RB1 and RB2 QTLs (Fazio et al., 2014). However, flowering time genes, FT-like genes, which were upregulated in the vasculature of apple dwarfing rootstocks, did not map to any major QTL associated with rootstock induced dwarfing (Foster et al., 2014). This indicates that the upregulation of flowering genes is not a primary effect but secondary consequences (Foster et al., 2014). All these studies suggest different mechanisms which could be controlling the rootstock-induced dwarfing. However, the genetic basis of dwarfing remains unknown.

Root architecture

Root system architecture (RSA) can be described as the spatial distribution of roots (Lynch, 1995; Osmont et al., 2007). RSA is essential for crop yield since it contributes to plant hydraulics, anchorage and nutrient uptake (Bohn et al., 2006; Lynch, 2007; Paez-Garcia et al., 2015; Ludlow and Muchow, 1990)**.**

Root systems have a great plasticity and root development is modified by a wide range of factors (Sultan, 2003). Root systems with a large number of ramifications at the top of the system can improve the uptake of immobile nutrients like phosphate (P) that remain at the topsoil (Lynch and Brown, 2001; White et al., 2013). Several studies in Arabidopsis have shown that lateral root and hair elongation are promoted under low levels of different nutrients, such as phosphate (P) (Bhosale et al., 2018)**,** iron (Fe) (López-Bucio et al., 2003) and boron (B) (Takano et al., 2006; Martín-Rejano et al., 2011; Abreu et al., 2014)**.** Similarly, in the common bean, secondary root growth is reduced under low phosphate whereas the root length increases, improving the phosphorus acquisition and yield (Strock et al., 2018). On the other hand, those root systems with less branching and deeper roots can forage for water

easily, reducing water stress, and providing better soil anchorage (Jordan et al., 1983; Lynch and Brown, 2001; White et al., 2013). All these root growth processes are mediated by complex hormone interactions. Cytokinins (CK), in the presence of auxins like indole-acetic-acid (IAA), regulate root development, vascular differentiation and lateral root initiation (Aloni et al., 2006). Whereas auxin (AUX) production and transport influence the lateral root development (Ljung et al., 2005; Petersson et al., 2009; Ljung, 2013), cytokinins can inhibit root formation (Werner et al., 2001; Werner et al., 2003). On the other hand, strigolactones (SL) increase root hair elongation, reduce lateral root formation and inhibit adventitious roots (Rasmussen et al., 2013).

Apple rootstocks have shown differences in RSA in commercial varieties. Ma et al., (2013) revealed that dwarfing rootstocks had the root system distributed in a small region while vigorous rootstocks had larger and deeper roots. Moreover, a later study manifested that the scion is also having an effect on the RSA (Harrison et al. 2016a).

A recent study identified 25 QTLs for root angle, a trait that is associated with root anchorage, using hybrids from the apple rootstocks "Baleng Crab" and M9. None of the QTLs overlap with the dwarfing QTLs suggesting that root angle could be related to dwarfing but not genetically linked (Zheng et al., 2020). Fortunately, more attention is being paid lately to root systems although further studies are still needed to investigate the genetic basis of the root spatial distribution in this perennial crop.

Nitrogen uptake

Nitrogen (N) is one of the most important nutrients for plant growth. It is part of amino acids and nucleic acids and is also an important signaling molecule needed for a large number of plant processes including lateral root growth, resistance to biotic and abiotic stress and mediation of hormone signaling (Wang et al., 2002; Vidal et al., 2010; Alvarez et al., 2012; Xu et al., 2012)**.** Nitrogen can be absorbed as nitrate and ammonium, although it can also be used in its inorganic forms, amino acids, proteins and peptides (Näsholm et al., 2009; Tegeder and Rentsch, 2010).

Nitrogen deficiency impacts plant metabolism and growth (Epstein, 2005). This leads to a reduction of biomass and therefore, a decrease in yield resulting in a serious problem for growers. To prevent N deficiency, nitrogen fertilizers are applied to the vast majority of crops. In 2015, more than 109 tonnes of nitrogen fertilizer for agricultural use were applied in the world (FAOSTAT 2018) although not all the N is efficiently used by the crops. Weinbaum showed that only 20% of the N fertilizer applied is used by fruit trees (Weinbaum et al., 1992). The remaining N is accumulated on the ground causing leaching and water contamination,

leading to serious environmental problems (Vitousek et al., 1997). Understanding the nitrogen use efficiency in crops could contribute to the improvement of the nitrogen fertilizer management through the development of more appropriate fertilization protocols for growers (Keeney, 1982).

Project aims

The main aim of the project is to understand the genetic mechanisms controlling dwarfing in apple rootstocks and how this affects root architecture, morphology (root bark percentage) and physiology (N uptake). The project has been divided into four main aims with different tasks within each aim to address the different challenges.

1. Root morphology on dwarfing apple rootstocks: Root bark percentage is greater in dwarfing rootstocks, but the mechanisms involved in this phenomenon are unknown. The main aim of this work package is to identify when this differentiation starts and what other differences exist in the root cells to better understand the physiological mechanisms that contribute to this complex phenomenon.

2. Understanding the genetic basis of dwarfing: the main aim of this work package is to fine map the root bark QTLs and identify potential genes involved in rootstock induced dwarfing which will help us to better understand this phenomenon.

3. Assess variation in root system architecture in different dwarfing classes. Previous studies at NIAB EMR showed that dwarfing has an impact on root architecture. The main objective is to assess variation in root system architecture within different dwarfing classes and ultimately to map major root traits.

4. Associate root type with nitrogen uptake capacity. Short term uptake of 15N stable isotope will be studied in dwarfing and vigorous rootstocks to determine if there are any differences in nitrogen uptake capacity (these experiments have been postponed due to Covid19).

Materials and methods

Effect of dwarfing on root morphology

Plant material

Four commercially available rootstocks with different degrees of dwarfing, M27, M9, M116 and MM106 (very dwarfing, dwarfing, semi vigorous and semi vigorous, respectively) were used for this experiment. Details provided in previous report.

Root embedding

Root samples were washed four times for 15 minutes with 0.1M phosphate buffer pH 7.2. After this, the root samples were dehydrated in the ethanol series (10%, 30%, 50%, 70%, 90% and 100%) for at least 30 minutes in each step. Samples were incubated at either 70% or 100% ethanol overnight at room temperature. Plastic infiltration was performed on the following day using the Technovit 7100 embedding kit. Root samples were submerged for one hour in a series of Solution A (100 ml Technovit 7100, 1 g Hardener I, 2.5 ml PEG 400) and 100% ethanol (Solution A:100% Ethanol series → 1:3, 1:1, 3:1) with a final step of 100% solution A overnight. On the following day, the polymerization solution was prepared by mixing 15 ml of Solution A and 1 ml of Hardener II. Mounting holders were placed on the plastics blocks and polymerization solution was added. Then, roots were carefully placed in the holders, making sure that the roots were touching the bottom of the blocks and staying upright. Polymerization was completed overnight at room temperature (Figure 2).

Figure 2. Embedded root in the plastic holder.

Sectioning, staining and microscopy

Sectioning was performed using the Brunel YD Rotary microtome. Several 10 µm cross sections were placed in a microscopy slide using tweezers and a pencil brush. Then, the slide was left in a hot plater for 10 minutes or until the sections were fixed to the slide. Staining was done using 0.05% Toluidine blue for 1 minute and then rinsed with distilled water. A drop of 50% glycerol was added to every slide and a glass coverslip placed on top to preserve the sections. The coverslips were sealed using Coverslip Sealant.

Sections were examined under the microscope using the Leica AF6000 imaging system and two measurements of the root and pith radius were recorded for each sample (Figure 3).

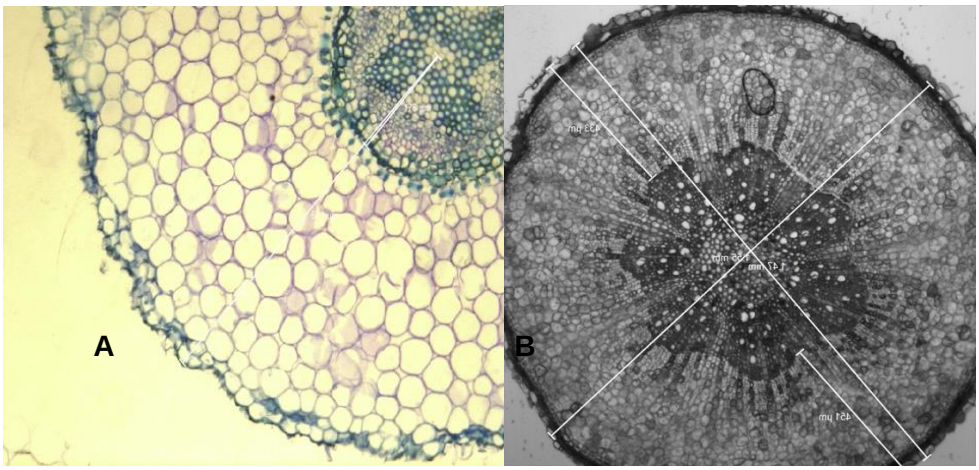

Figure 3. Root sections stained with 0.05% Toluidine blue. A. M116 root section. B. M27 root section.

Data analysis

Statistical analyses and graphical outputs were performed using the statistical software package 'Rstudio' version 3.4.0 using the lme4 package (R core development Core Team, 2017). The models presented were developed by sequentially adding fixed effects to the regression analysis, using single additive factors and interaction terms. Final models were selected, based upon significant terms in the model selection using a one-way ANOVA. To determine significant differences between means, Tukey's honest significant difference test (TukeyHSD) was carried out using the emmeans package available in RStudio.

Understanding the genetic basis of dwarfing

Summary of markers

Highly polymorphic SSRs markers spanning the three genetic regions in chromosomes 5, 11 and 13 associated with root bark percentage (Harrison et al. 2016) were identified. These

regions were used as a target for primer design using Primer3 software (Untergasser et al., 2012) available in Geneious. The best working SSR primers were then tested on known germplasm (a M27xM116 cross) to identify which of the two alleles in each locus is the dwarfing allele. Two multiplexes were prepared with the best working markers and screened in 7 strategic rootstock crosses that segregated for vigour (see details in the previous report).

Canopy and root bark measurements

In December 2019, height and trunk diameter were measured in the 335 trees that were still alive. Three to ten root segments (2–8mm in diameter, 50–80mm in length) were excised from each root system using secateurs, placed into a labelled polythene bag and stored at 4°C before analysis. The roots were then carefully washed using tap water. For each root segment, a scalpel or knife was used to remove a ring of bark (cortex) approximately 2–3mm in length, leaving behind the stele of the root (Figure 4). Digital calipers were used to make pairs of measurements of the root with and without the bark. The cross-sectional area of the root and the percentage of total area occupied by the root bark were calculated for each sample, assuming that the root section was a perfect cylinder. Trees were carefully planted back in their pots to keep the plant material in case new measurements are needed.

Figure 4. Root segments after bark removal. Red arrows pointing the area where bark was removed.

Data analysis using MCM families

MCM001 and MCM007 non-recombinant genotypes were classified according to the presence or absence or the dwarfing haplotype and the number of copies of the dwarfing haplotype in each region. Root bark percentage was normalised using an inverse square transformation.

This information was used to analyse the effect of each QTL in the root bark in this dataset with multiple families.

For the fine mapping analysis some recombinants from MCM006 family were excluded from the analysis since it was not possible to know if the haplotype coming from M26 was dwarfing or not due to homozygosity in markers in region 5. All the roots collected were used in this analysis. Recombinants were grouped depending on which markers they have lost or conserved during recombination events. A selection of fully dwarfing and completely vigorous genotypes were used for reference in the fine mapping analysis.

Statistical analyses and graphical outputs were performed using the statistical software package 'Rstudio' version 3.4.0 using the lme4 package (R core development Core Team, 2017). The linear mixed models presented were developed by sequentially adding fixed effects to the regression analysis, using single additive factors and interaction terms. Final models were selected, based upon significant terms in the model selection using a one-way ANOVA. To determine significant differences between means, Tukey's honest significant difference test (TukeyHSD) was carried out using the emmeans package available in RStudio.

Breeding programme trials

In April 2018, three apple trials from the rootstock breeding programme in EMR were grubbed using a digger (Table 1). These rootstocks had a dwarfing rootstock as a parent or grandparent; therefore, the dwarfing haplotype could be present in them and could contribute to the fine mapping of the root bark QTLs and to the validation of the dwarfing markers in different germplasm. Trees in trial A were only grafted with Gala and trees in trial B were grafted with Red Falstaff. However, trees in trial C were grafted with Braeburn or Royal Gala. Roots were collected from all the rootstocks available. Eight to fifteen root segments (4–10 mm in diameter, 80–120 mm in length) were excised from each root system using secateurs, placed into a labelled polythene bag and stored at 4°C before analysis. The roots were measured following the same protocol described above.

Table 1. Details of the rootstocks from trials A, B and C for fine mapping the root bark QTLs.

Trial name	Planting year	Genotype	Pedigree	Number of trees harvested
A	2012	M9	Unknown	4
A	2012	MM106	Northern Spy x M1	4
A	2012	M116	M27 x MM106	4
A	2012	M306-6	AR86-1-20 x M20	4
A	2012	M306-20	AR86-1-20 x M20	4
A	2012	M306-79	AR86-1-20 x M20	4
A	2012	M306-189	AR86-1-20 x M20	4
B	2010	AR10-3-9	MM106 x M27	7
B	2010	AR809-3	R80 x M26	8
B	2010	AR835-11	M793 x M9	7
B	2010	M116	M27 x MM106	8
B	2010	MM106	Northern Spy x M1	8
B	2010	R80	AR134-31 x AR86-1-22	8
C	2010	AR852-3	AR362-16 x OP	11
C	2010	AR839-9	M7 x M27	15
C	2010	B24	AR-10-2-5 x AR86-1-22	10
C	2010	M26	M16 x M9	15
C	2010	M27	M13 x M9	17
C	2010	M9	Unknown	17
C	2010	R104	AR134-31 x AR86-1-22	10
C	2010	R59	AR134-31 x AR86-1-22	15

In May 2020, another apple trial containing Canadian and Malling rootstocks was grubbed and roots were collected (Table 2). Trees were grafted either with Gala or Braeburn. Four trees were collected from each rootstock/scion combination when possible. Six to eighteen root segments (4–10 mm in diameter, 80–120 mm in length) were harvested from each root system and processed as described in the above paragraph.

Table 2. Details of the rootstocks harvested from trial D for fine mapping the RB QTLs.

Trial name	Planting year	Genotype	Pedigree	Number of Gala trees harvested	Number of Braeburn trees harvested
D	2014	SJM167	M. Baccata 'Nertchinsk' x M9	4	4
D	2014	SJM188	M. Baccata 'Nertchinsk' x M9	4	4
D	2014	SJM189	M. Baccata 'Nertchinsk' x M9	4	4
D	2014	SJP84-5162	M. Robusta 5 x M26	4	4
D	2014	SJP84-5174	M. Robusta 5 x M27	4	4
D	2014	SJP84-5217	M. Robusta 5 x ?	4	4
D	2014	SJP84-5231	M. Robusta 5 x ?	4	4
D	2014	M26	M16 x M9	4	4
D	2014	M9	Unknown	4	4
D	2014	MM106	Northern Spy x M1	4	4

DNA from most of the rootstocks was available and was extracted using Qiagen Dneasy Kit by Suzanne Litthauer, the assistant molecular breeder working in the rootstock breeding programme. DNA was diluted to 5ng/ul and the first primer multiplex was screened in all the rootstocks following the protocol described in Section 5.2.2. Next, the other two multiplexes with more markers for chromosome 5 and 11 regions were screened in the correspondent recombinants (see details in Section 5.2.3) to narrow down a bit where the recombinations are taking place.

Breeding trials data analysis

The number of roots measured in each genotype was higher than in other progenies. Therefore, the percentage of root bark at a standard root diameter of 7.5 mm was inferred using regression analysis.

Recombinant rootstocks were identified in Trial A and D. An individual statistical analysis was done for each trial. In trials where rootstocks were grafted with two scions, the analysis was individually done for each variety. Statistical analyses and graphical outputs were performed using the statistical software package ‘Rstudio’ version 3.4.0 using a similar approach as described above in the MCM families data analysis (see details above).

NH apple rootstock families

In order to find more recombinants in older trees to aid in the fine mapping of the root bark QTLs, new populations were examined. NH006, NH007 and NH008 families were germinated in March 2015 and planted in the field in June in 2016 at Deadman plot in NIAB EMR by Magdalena Cobo Medina while working as a research technician (Table 3).

Table 3. Details of the new crosses used for fine mapping the RB QTLs.

Cross name	Pedigree	Number of individuals
NH006	M116 x M27	25
NH007	M27 x M116	36
NH008	M13 x M9	31

In May 2020, leaf material was collected from all the individuals of these families and DNA was extracted using the Silica Bead Method (SBM) described in (Edge-Garza et al. 2014).

DNA was quantified using Nanodrop and then diluted to 5 ng/ul. Next, the first batch of primers were screened in all the individuals and later, the other two multiplexes with extra markers for chromosome 5 and 11 regions were also tested on the correspondent recombinants following the protocols above described in sections 5.2.2 and 5.2.3 respectively.

Roots will be collected during winter 2020 from the recombinant genotypes and the statistical analysis will be then performed.

Effect of dwarfing on root system architecture

Previous work

As detailed in the previous report, DNA from all the individuals of the MDX132 mapping population (Golden Delicious x M9 cross) and from the parents was extracted. Next, 150 individuals and the parents were genotyped using the Illumina Infinium® SNP array.

SNP calling was performed using GenomeStudio Genotyping Module 2.0 (Illumina) and the filtering was done using the software ASSisT (Di Guardo et al., 2015). Finally, a linkage map was produced with JoinMap 4.1 (see details in previous report) which will be used, together with the root data extracted from the rhizotrons images, to map important root traits.

Lifting stoolbeds and rooting phenotyping in 2020

An existing mapping population is being used for this chapter experiment. The MDX132 population (Golden Delicious x M9) consists of 287 individuals of which 240 were planted in Deadman plot at NIAB EMR in 2016.

Rootstocks were stooled in June 2018 to conduct the experiment in 2019 but unfortunately not many rootstocks produced well rooted shoots and therefore, the experiment was postponed until 2019/2020.

In spring 2019, the trees were cut back to ground level to induce branching. In mid-June 2019, most of the genotypes had produced several new shoots and were earthed up using bottomless 10 litre pots. Pots were filled with moist sawdust in order to cover about 4-5 inches of the shoots and the remaining 2 inches were filled up with soil.

In January 2020, stoolbeds were carefully unearthed and rooted shoots were labelled and stored at 4 °C for further experiments about root system architecture. Number of total shoots, rooted shoots, root length and root quantity were recorded for each genotype (Figure 5).

Figure 5. Lifting stoolbeds process. A. Pots removed. B. Shoots carefully unearthed. C. Phenotyping of rooted shoots from a particular genotype.

Genotype selection for root architecture experiment

SNPs flanking RB1 and RB2 regions were identified in the current linkage map and according to this, the individuals with no recombinations in these areas were divided in four groups (Table 4). Then, well rooted genotypes from each group were selected for the experiment. The initial idea was to use replicates per genotype but unfortunately not many genotypes had several well rooted shoots, therefore, in this experiment there are no replicates within genotypes. Trunk diameter was recorded for the selected genotypes and photos of the initial roots were also taken to be included in the analysis.

Table 4. Number of genotypes in each dwarfing class and the predicted vigour.

RB1 haplotype	RB2 haplotype	Number of genotypes	Predicted vigour
No	No	9	Vigorous
Yes	No	10	Vigorous
No	Yes	9	Vigorous
Yes	Yes	12	Dwarfing

Root system architecture rhizotrons

Selected genotypes were grafted at the end of March using Gala graft wood collected from a single Gala tree available at the NIAB EMR. Afterwards, grafted trees were planted in rhizoboxes (100cm x 30cm x 3cm) filled with sieved standard compost without slow release fertilizer. The slow release fertiliser can be confused with roots due to its whitish colour. Rhizoboxes were covered with white reflective plastic to prevent roots from direct light.

The rhizotrons were randomized in 4 blocks and placed in an environmentally controlled glasshouse compartment (Figure 6) with an inclination of approximately 15°. Trees were fertigated twice a day for 2 minutes at 8 am and 4 pm using a Dosatron with Universol Green 23-6-10 (N-P-K) fertilizer.

Figure 6. Rhizotrons to assess the effect of dwarfing on RSA in the glasshouse compartment.

Canopy phenotyping and imaging

Rhizotrons imaging and canopy phenotyping (trunk diameter and height) took place every six weeks from June until October. A homemade imaging rig was prepared with 2 Canon 1200D cameras with an 18-55 mm telephoto (using the 18 mm) on a camera slider. The total length of the rhizotron was covered by overlapping the two images.

The imaging platform consisted of a Dexion frame where the rhizoboxes were positioned at a fixed distance from the camera. The whole imaging structure was covered by a black cloth and two Manfrotto LED lighting units were used to minimize the variation of the ambient light as much as possible. Images were taken at an f stop of 5.6 to 6.3 at 1/60 using a shutter release.

Final phenotyping

The last imaging and canopy phenotyping were performed in October 2020. Then, the trees were cut at the graft union level and the canopy was weighed. Root systems were carefully washed with tap water to get rid of the soil and the root systems were also weighed (Figure 7). Ten to fifteen root segments (2-8 mm in diameter, 50-80 mm in length) were excised from each root system using secateurs, placed into a labelled polythene bag and stored at 4°C before analysis. Digital callipers were used to make pairs of measurements of the root with and without the bark as described in the previous section. The percentage of root bark at a standard root diameter of 7.5 mm was then inferred in each genotype using regression analysis. Data analysis is ongoing.

Figure 7. Washed root systems ready to be weighted.

Imaging analysis

Lens distortion and colour correction of the images is being done using RawTherapee 5.8. Then, the two photos of each rhizotron are stitched together using the Fiji plugging available in ImageJ (Schindelin et al. 2012). Afterwards, the image segmentations and the root measurements will be done using some specific python scripts developed by Pen Pennington while he was working at NIAB EMR in 2016. Finally, data analysis will be performed using 'Rstudio'. Imaging analysis is still ongoing.

Results and discussion

Effect of dwarfing on root morphology

Samples from the third time point were the first processed roots. No relevant results were found at this time and therefore, samples collected during the first and the second point were not processed.

In this analysis, the best fitting model did not find a statistically significant effect of root size on root bark percentage. Surprisingly, all rootstocks had a high percentage of root bark in the roots collected during the third time point, 5 months after planting. This revealed that all rootstocks start with a large percentage of bark, and as they develop, the proportion of bark that the vigorous rootstocks have will decrease with respect to the dwarfing rootstocks (Figure 8).

No significant differences between rootstocks were found at this stage. At this stage, MM106 rootstocks showed the highest root bark percentage average (81.7%), which is the opposite to what we expected. The great variety of sizes in the collected roots as well as the low number of replicates may be influencing this result.

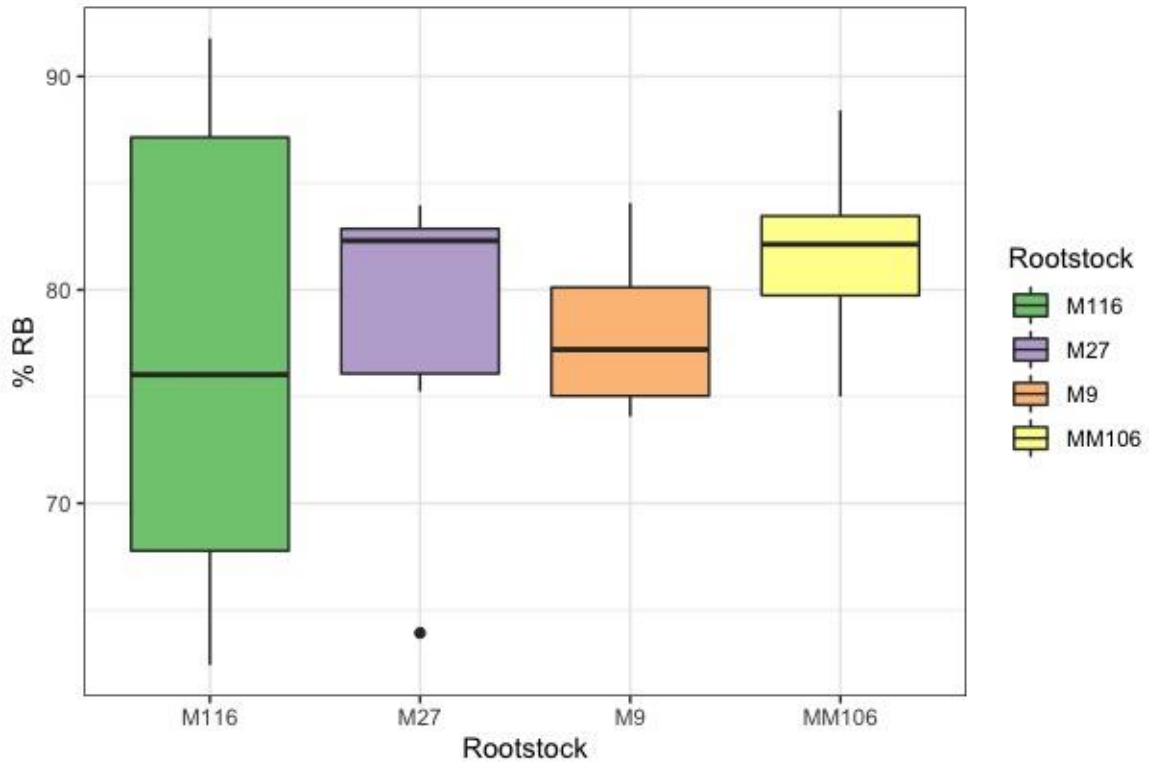

Figure 8. Box plot showing the variation in root bark percentage in rootstocks after 5 months planted. The median is shown as a thicker dark line.

By October, seven months after planting, some significant differences were already detected. M27 was the rootstock with the highest root bark percentage average (83.2 %) and MM106 the rootstock with the smallest percentage as expected (79.2%). There is a huge variation in M116, probably due to the great variability in root sizes. Significant differences were found between M27 with MM106 (*P=0.0077*) and with M116 (*P=0.03*). No significant differences were found between M9 and the vigorous rootstocks yet (Figure 9).

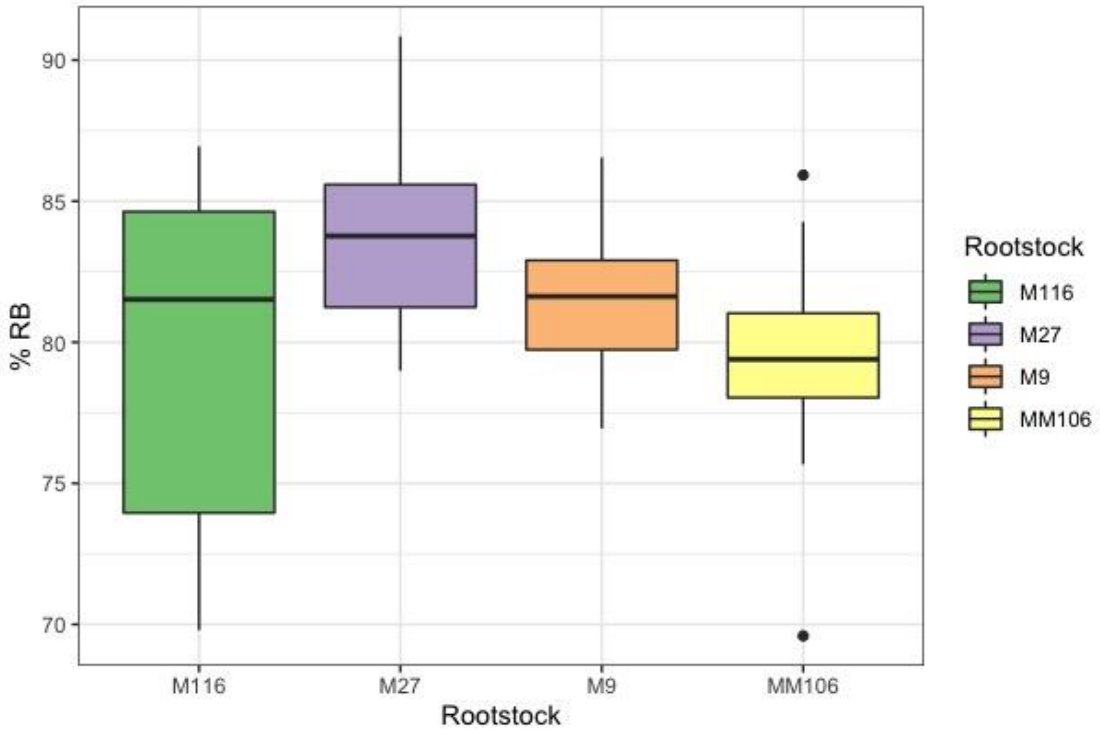

Figure 9. Box plot showing the variation in root bark percentage in rootstocks after 7 months planted. The median is shown as a thicker dark line.

By this time, root diameter was close to being statistically significant (*P=0.06*) suggesting that smaller roots have a high root bark percentage regardless of the variety. Different size roots cannot be compared since they could be at a different development stage. Due to the low number of rootstocks available for the experiment, the roots harvested in each time point were carefully selected and not many roots were collected since this could kill the trees. A greater number of similar size roots need to be collected across all the rootstocks to determine when roots from dwarfing and vigorous rootstocks are different in root bark percentage and also to evaluate the effect of root diameter in root bark.

Knowing that initially all rootstocks had a high root bark proportion, my hypotheses were reformulated. One hypothesis is that vigorous rootstocks develop much faster than dwarfing rootstocks and therefore, the percentage of root bark decreases more quickly in the vigorous roots. Another hypothesis is that vigorous rootstocks could be increasing the amount of xylem in at the expense of root bark.

Future experiments to test these hypotheses are required to help us to understand the differences in morphology between dwarfing and vigorous rootstocks.

Understanding the genetic basis of dwarfing

Recombinants in MCM rootstocks populations

Using the dwarfing haplotype previously identified, recombinants in chromosome 5 and 11 were detected (Table 8).

A total of 55 recombinants were found in chromosome 5, and 87 recombinants in the chromosome 11 region. The expected number of recombinants was much bigger but unfortunately the number of outcrosses in 4 families was very high and not many recombinant individuals could be detected in those populations (Table 5).

Table 5. Number of outcrosses and recombinants genotypes detected in the rootstock populations after screening the first batch of markers for fine mapping the RB QTLs.

Family name	Female	Male	Number of seedlings	% outcrosses	Final number of seedlings	Total number of recombinants	Rec in Chr 5	Rec in Chr 11	Rec in both regions
MCM001	M9	M27	42	9%	38	22	4	16	2
MCM002	M27	M26	98	81%	18	20	8	11	1
MCM003	M116	M27	184	75%	37	10	3	5	2
MCM004	M27	M116	38	94%	2	6	2	4	0
MCM005	M9	M26	34	88%	3	6	0	6	0
MCM006	M26	M27	140	30%	98	39	15	20	4
MCM007	MM106	M26	335	49%	168	51	23	25	3
Total			871		364	154	55	87	12

The percentage of outcrosses in MCM001 is 9%. In every cross there are always some seeds that do not belong to the cross due to pollination problems. A total of 22 genotypes with recombinations were detected in this progeny.

In MCM002 cross, the percentage of outcrosses was 81%, some of them did not even have any of the maternal haplotypes, meaning that probably some seeds from other crosses or wrong apples were mixed with this population. However, we managed to find 20 recombinants in the progeny of this cross and in those outcrosses conserving the dwarfing maternal haplotype. In the latest case, the most likely explanation is that some flowers were not fertilised with our M26 pollen and another pollen that we do not recognise fertilized the flowers instead.

In the MCM003 family, the percentage of outcrosses accounted for 75%. A total of 10 recombinants were found in the progeny of this cross. Although most of the outcrosses conserved one of the maternal haplotypes, recombinants were not found in chromosome 5 region since M116 does not have the dwarfing haplotype and surprisingly not many individuals had recombinations in the region of chromosome 11 where M116 has the dwarfing haplotype.

In MCM004 and MCM005 the percentage of outcrosses was extremely high, 94% and 88% respectively, probably due to errors in the pollination process or poor quality of the pollen. It was possible to find recombinants in both crosses since some of the outcrosses conserved the maternal haplotype, and M27 and M9 have the dwarfing haplotype in both regions. A total of 6 recombinants were identified in each family.

Only 30% of the seedlings were outcrosses in the MCM006 population. A total of 39 recombinant individuals were detected in this family. The number of recombinants detected in this cross is a bit smaller than expected since most of the markers in chromosome 5 region in M26 are homozygous and it is not possible to distinguish when there is a recombination process in this area.

Finally, in the MCM007 family, 49% of the individuals did not belong to this cross. Outcrosses in this case were not useful since MM106 does not have the dwarfing haplotype in any region. A total of 51 recombinants were identified in the progeny.

Useful recombinants in MCM rootstocks populations

Unfortunately, not all the recombinants detected, detailed in the previous section, are useful for fine mapping these QTLs. Both of the dwarfing haplotypes are needed to cause the dwarfing phenotype (Harrison et al. 2016b). Therefore, recombinant genotypes only in one region were discarded for this analysis. In addition, only one copy of the dwarfing haplotype

is needed to cause dwarfing. Consequently, genotypes with two copies of the dwarfing haplotype, one of them with a recombination, were also discarded since the effect of the loss of a dwarfing region cannot be evaluated. Genotype recombinants in both regions at the same time are not useful since if the dwarfing phenotype is lost it would be impossible to know which of the two recombinations is responsible for the phenotype. Some of these recombinants could be useful once the regions are narrowed down. The final number of useful recombinants is 24 individuals in the region in chromosome 5 and 33 genotypes in chromosome 11 region (Table 6).

Table 6. Number of useful recombinants in each rootstock population.

Family name	Female	Male	Number of useful recombinants in Chr5	Number of useful recombinants in Chr11
MCM001	M9	M27	1	3
MCM002	M27	M26	3	6
MCM003	M116	M27	2	2
MCM004	M27	M116	1	0
MCM005	M9	M26	0	1
MCM006	M26	M27	5	11
MCM007	MM106	M27	12	10
TOTAL			24	33

Tree height and % RB correlation

Many studies about dwarfing have measured trunk diameter, tree height or flower density as a trait to evaluate the level of dwarfing. My study does not show a clear correlation between tree height and root bark percentage. In Figure 10A using all the roots harvested there is a small negative correlation between these two traits (-0.08). This correlation slightly improves when only roots bigger than 3 mm are used in the analysis (-0.24) (Figure 10B). Tree height is influenced by many factors; therefore, not finding correlation between root bark percentage and tree height is not unexpected. However, the fact that correlation improves when small roots are excluded from the analysis suggests that small roots are altering the results and that this correlation will be stronger using thicker roots.

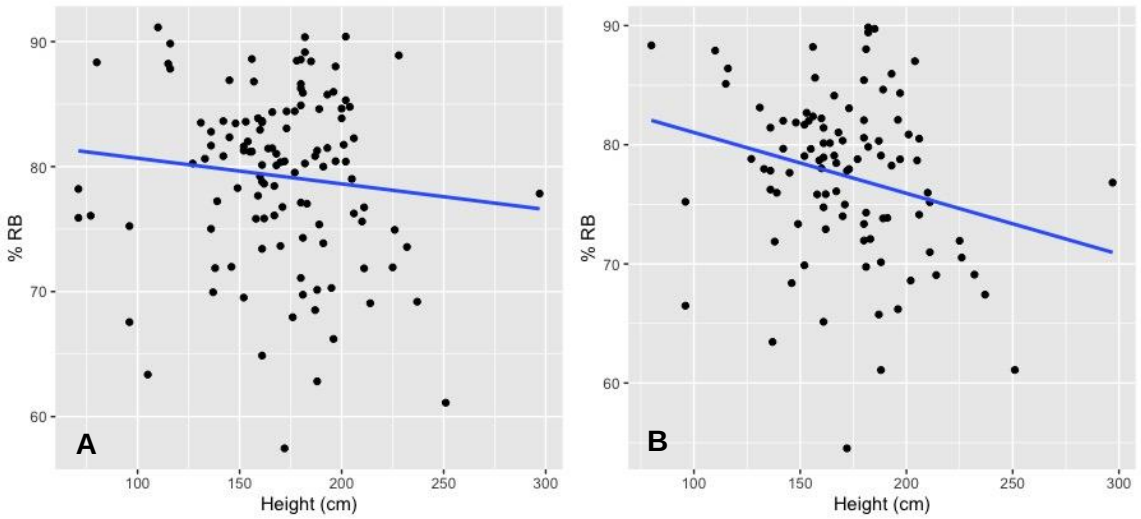

Figure 10. A. Spearman's rank correlation between height and % RB including all size roots (-0.08). B. Spearman's rank correlation between height and % RB including only roots bigger than 3 mm (-0.24).

Trunk diameter and % RB correlation

A previous study established a strong correlation between trunk diameter, the most commonly used trait to measure the level of dwarfing, and percentage of root bark (Harrison et al. 2016b). However, no correlation was found in this data set (Figure 11). As with tree height, trunk diameter may be influenced by other factors, but some correlation was expected. This correlation also showed that the data set is not behaving as expected.

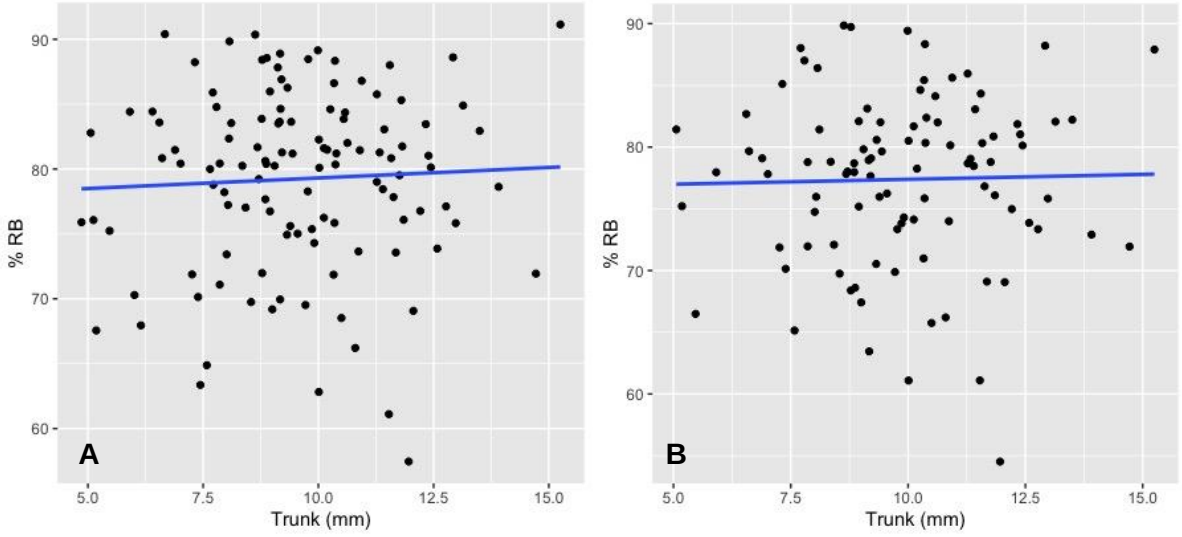

Figure 11. A. Spearman's rank correlation between trunk diameter and % RB including all size roots (0.031). B. Spearman's rank correlation between trunk diameter and % RB including only roots bigger than 3 mm (0.023).

Effect of each QTL in the root bark phenotype

Unfortunately, the roots collected from MCM populations for the fine mapping were in general too small as the trees were younger than 2 years. In addition, not many roots could be collected from most of the genotypes, therefore, the percentage of root bark at a standard root diameter of 7.5 mm could not be inferred using regression analysis.

The best fitting model included root diameter, RB1 and RB2 as fixed effect and the genotype as random effect. All the effects were significant as well as the interaction between RB1 and RB2. Root diameter was highly significant in the model, confirming what we have observed in the root bark development experiment, that smaller roots have a high root bark percentage (Table 7).

Table 7. Type I Analysis of Variance Table with Satterthwaite's method showing the p values of the fixed effects. Sequentially adding terms to model.

Fixed effect	Sum sq	Mean sq	F value	Pr(>F)
Root diameter	52.213	52.213	154.7533	2.2e-16 ***
RB1	6.156	6.156	18.2456	9.053e-06 ***
RB2	4.398	4.398	13.0358	0.0002099 ***
RB1:RB2	1.394	1.394	4.1323	0.0445240 *

The data shows the expected trend but with much higher root bark values than previously seen by Harrison et al., (2016b). Significant differences were detected between genotypes with both RB QTLs and genotypes with no dwarfing QTLs (*P<0.0001*). Also, significant differences were found when comparing genotypes with both QTLs, RB1 and RB2 against genotypes with only one QTL, either RB1 (*P=0.0005*) or RB2 (*P<0.0001*). No significant differences were found between genotypes with only one QTL and vigorous genotypes with no dwarfing QTLs. This confirms that both QTLs are needed to cause the dwarfing phenotype (Figure 12).

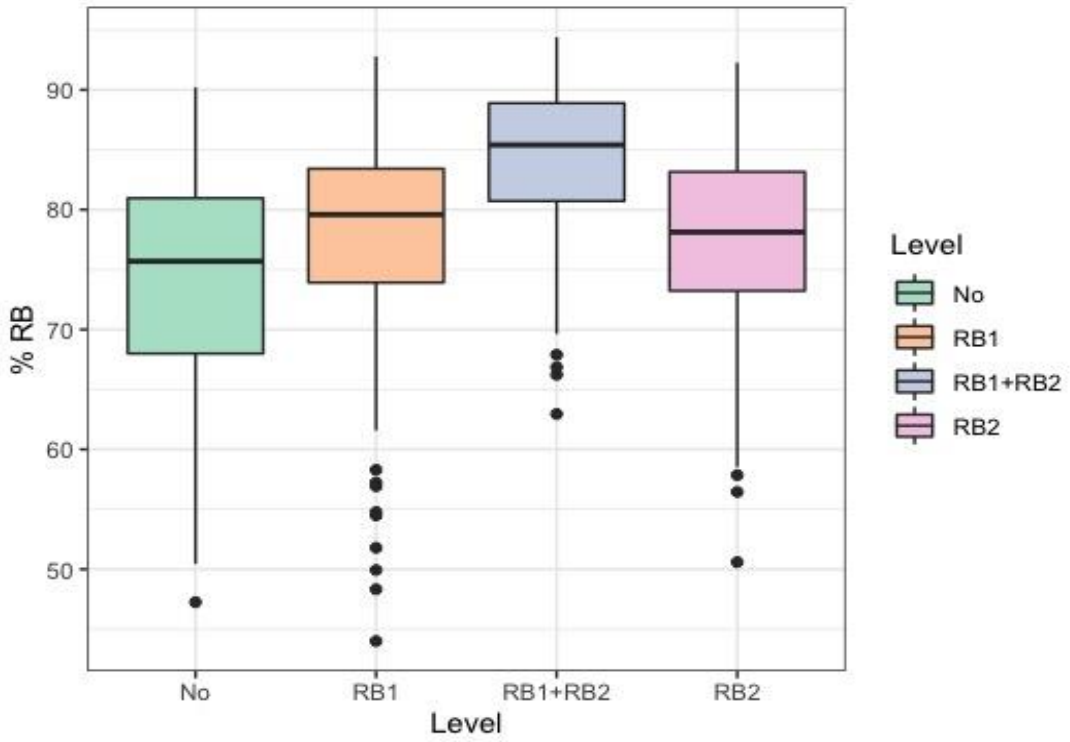

Figure 12. Box plot depicting the effect of RB QTLs in root bark percentage using all size roots. The median is shown as a thicker dark line.

Fine mapping RB1 using MCM families

The number of recombinant genotypes in each recombinant group was very small (Figure 13). The low number of roots and replicates within each group makes the analysis quite complicated and reliability of the data a bit low.

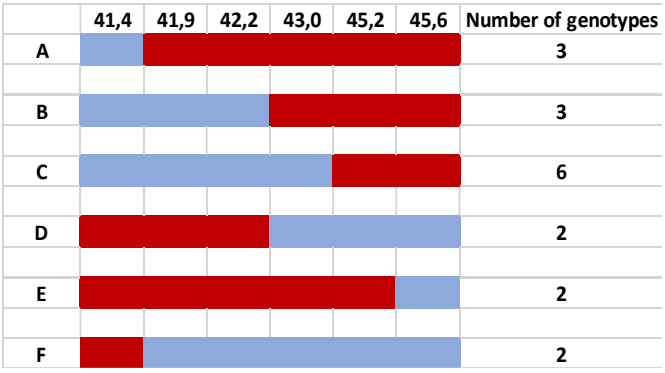

Figure 13. Drawing showing the group of recombinants and the number of recombinants in each group. In blue, dwarfing allele present.

In this analysis, the best fitting model includes genotype as random factor and root size and group (recombinants and controls) as fixed effect (Table 8). Significant differences were only found when comparing dwarfing control group with the vigorous control group (*P=0.035*). No differences were detected in other groups; therefore, it was not possible to fine map the root bark QTLs and more recombinants are needed.

Table 8. Type I Analysis of Variance Table with Satterthwaite's method showing the p values of the fixed effects. Sequentially adding terms to model.

Fixed effect	Sum sq	Mean sq	F value	Pr(>F)
Recombinant groups	12.586	1.798	3.1963	0.01181 *
Root size	21.135	10.567	18.7858	3.681e-08 ***

The recombination that occurred to generate the M27 rootstock manifested that a region at the bottom of the QTL is not needed to cause dwarfing since M27 did not inherit that region and is one of the most dwarfing rootstocks known. Therefore, markers located at 45.2 Mb and 45.6 Mb are no longer in the dwarfing region. It was noticed that Group E of recombinants looks more similar to the vigorous group. Group E recombinants have only one marker with the dwarfing allele located at 45.6 Mb and we already know that marker is not needed to cause dwarfing (Figure 14). Therefore, recombinants in Group E should be vigorous but this cannot be confirmed by statistical analysis.

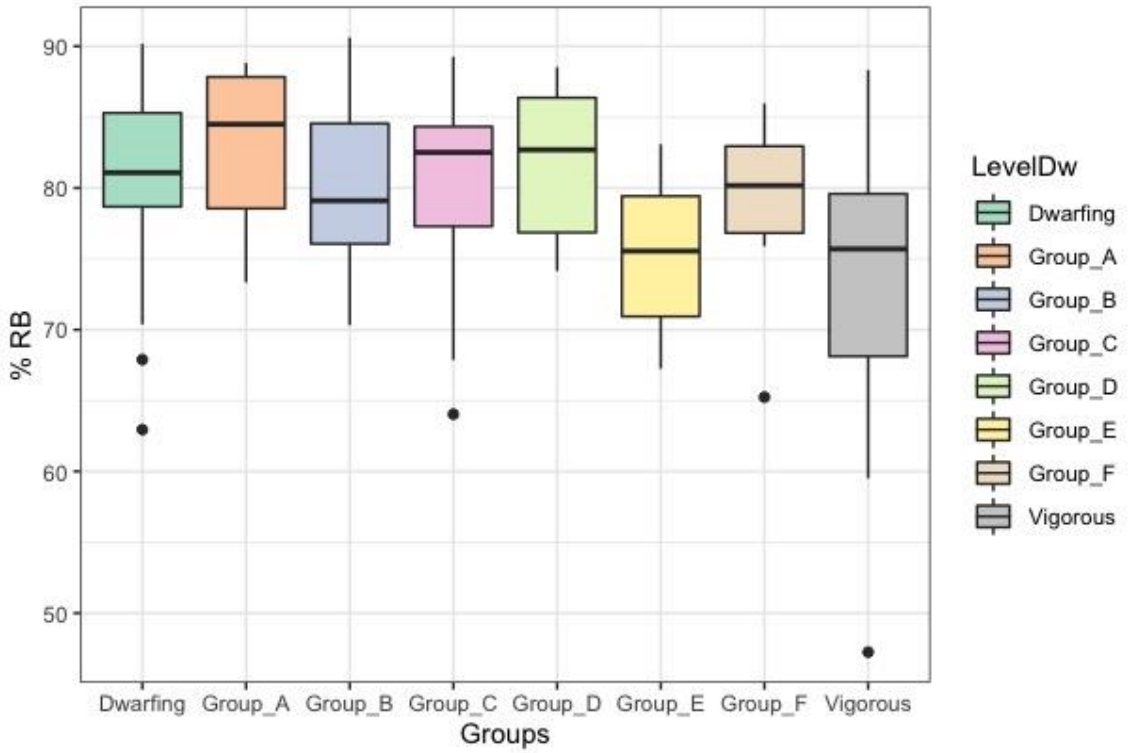

Figure 14. Box plot showing the average root bark percentage of the recombinant groups compared to the root bark percentage of a subset of fully dwarfing genotypes and a subset of vigorous genotypes. The median is shown as a thicker dark line.

Although some useful information has been collected from this analysis, more recombinants are needed to finally fine map the root bark QTLs.

A similar result was obtained when RB2 region was used for fine mapping and again, contradictory data did not allow the fine mapping of the region (data not shown).

Rootstock breeding program trial A

M116 and MM106, with 57.1% and 54.1% of root bark respectively, are both semi invigorating rootstocks used in this trial as controls. M9 is the only dwarfing rootstock used as control, with 70.1% of root bark percentage. M306-079 (59.4% of root bark) and M306-189 (66.1% of root bark) are recombinant genotypes and the control rootstocks were compared to these recombinants to evaluate the effect of the recombination in the dwarfing phenotype (Figure 15). The genotypes with no recombinations will be used to validate these markers and their ability to predict dwarfing.

		M306-079		M306-189	
Markers Chr 5	**Mbp**	**H1**	**H2**	**H1**	**H2**
MD5001	41,4	129	133	128	132
MD5002	41,9	201	205	201	205
MD5003	42,2	140	142	139	141
MD5006	43,1	245	235	245	235
MD5007	45,2	NA	NA	NA	NA
MD5004	45,6	251	251	253	251
Markers Chr 11	**Mbp**	**H1**	**H2**	**H1**	**H2**
MD11001	6,9	232	212	221	199
MD11004	7,5	210	203	198	205
MD11005	8,3	98	98	98	98
MD11002	9,8	141	141	140	147
MD11006	10,4	170	170	170	170
MD11007	10,9	155	155	155	155
MD11003	12,7	268	276	268	272

Figure 15. Marker details of the recombinant genotypes identified in trial A.

The best fitting model includes rootstock as fixed effect (*P=5.634e-06)* and block as random effect. No differences were detected when comparing M306-079 with the semi vigorous rootstocks M116 and MM106 (*P=0.84* and *P=0.19* respectively). Significant differences were found between M9 and M306-079 (*P=0.008*). This indicates that M306-079 looks more similar to semi vigorous rootstocks and it will be classified as semi invigorating rootstock (Figure 16).

Significant differences were found between M116 and M306-189 and between MM106 and M306-189 (*P=0.014* and *P=0.0017*). However, no significant differences were detected when comparing M306-189 with M9 (*P=0.59*) (Figure 16). This suggests that M306-189 can be classified as dwarfing.

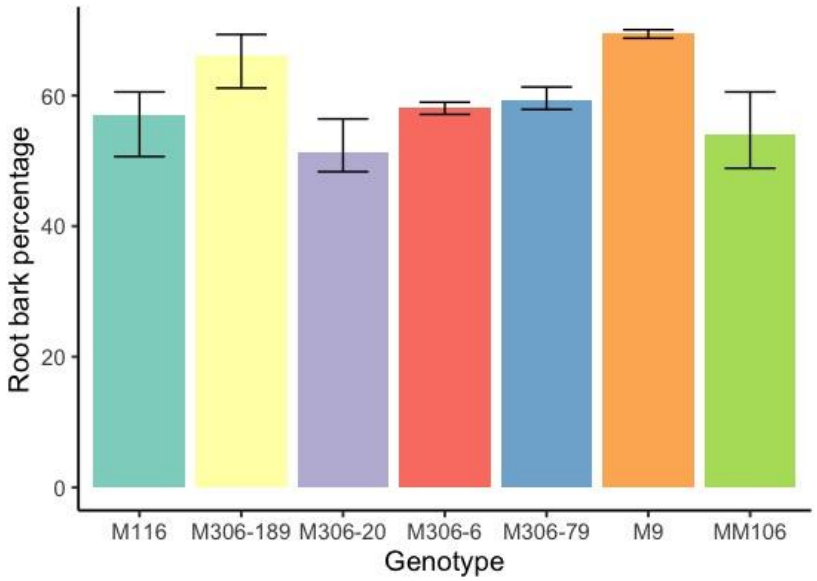

Figure 16. Effect of rootstock on root bark percentage in trial A.

Rootstock breeding trial D

In this trial, rootstocks were grafted with 2 scions; Braeburn and Gala. An independent analysis for rootstocks grafted with each scion was performed since significant differences in root bark proportion were detected between scions (see details in the next section). Recombinant rootstocks were compared against the dwarfing and semi vigorous rootstocks to determine to which group they belong.

In Braeburn rootstocks, MM106 with 51% of root bark is the semi vigorous rootstock used as control in this trial and M9 with 64.7% of root bark is the dwarfing control. In this trial, SJM167, SJM188 and SJP84-5174 are the recombinant genotypes with 51.4%, 56.4% and 62.3% of root bark respectively (Figure 16). The remaining genotypes will be used for markers validation.

		SJM167		SJM188		SJM189		SJP84-5174	
Markers Chr5	**Mbp**	**H1**	**H2**	**H1**	**H2**	**H1**	**H2**	**H1**	**H2**
MD5001	41,4	119	132	119	132	119	132	130	128
MD5002	41,9	199	205	199	205	199	205	NA	NA
MD5003	42,2	176	141	176	141	176	141	141	150
MD5006	43,1	263	235	252	235	252	235	247	235
MD5007	45,2	262	276	272	276	272	276	264	276
MD5004	45,6	236	251	247	251	247	251	251	251
Markers Chr11	**Mbp**	**H1**	**H2**	**H1**	**H2**	**H1**	**H2**	**H1**	**H2**
MD11001	6,9	212	212	221	212	222	212	195	212
MD11004	7,5	205	203	191	191	191	191	NA	203
MD11005	8,3	116	116	122	116	122	116	NA	128
MD11002	9,8	146	155	159	155	159	155	140	142
MD11006	10,4	168	168	174	168	174	168	NA	164
MD11007	10,9	161	158	168	158	168	158	NA	159
MD11003	12,7	266	266	266	266	266	266	262	272

Figure 16. Marker details of the recombinant genotypes identified in trial D.

The best fitting model included rootstock as fixed effect (*P=0.0002*) and planting row as random effect. As expected, there are significant differences between M9 and MM106, the reference rootstocks (*P=0.004*).

Significant differences were also found between M9 and SJM167 (*P=0.006*). However, there are not significant differences in root bark percentage between SJM167 and MM106, the semi vigorous control (*P=1.00*), suggesting that SJM167 can be classified as semi vigorous rootstock.

No differences were detected when SJM188 was compared to either M9 or MM106 (*P=0.19* and *P=0.60* respectively). Therefore, these results are inconclusive, and it does not allow us to classify SJM188 in any of the groups. This could be explained by the small number of roots

collected from this genotype. One replicate was removed from the analysis and the remaining trees did not have a big number of roots. However, if we observe the p values, SJM188 looks closer to be classified as vigorous rather than as dwarfing. This would make sense since SJM167 has a similar recombination pattern and it has been classified as semi vigorous.

No differences were observed between M9 and SJP84-5174 (*P=0.99*). Then, MM106 was also compared to SJP84-5174 and no significant differences were identified either (*P=0.051*). Although these differences are not statistically significant, the data suggests that SJP84-5174 is closer to be classified as dwarfing/semi-dwarfing rather than as semi-vigorous.

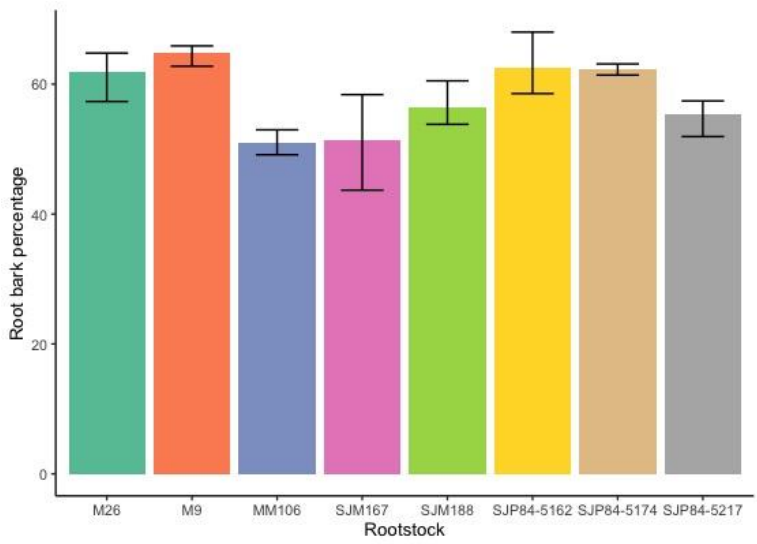

Figure 17. Effect of rootstock grafted with Braeburn on root bark percentage in trial D.

In rootstocks grafted with Gala, M9 has on average 73.5% of root bark. MM106, the semi invigorating control, has 58.3% of root bark on average. In this trial, the recombinant genotypes are, as in Braeburn, SJM167 and SJP84-5171, and instead of SJM189, SJM188 is the rootstock grafted with Gala.

The best fitting model includes rootstock as fixed effect (*P=6.491e-05*) and planting row as random effect. It is not surprising that significant differences were encountered when comparing M9 and MM106, with the control groups (*P=0.006*).

SJM167 and M9 contrast did not show statistically significant differences (*P=0.06*). SJM167 and MM106 comparison did not exhibit differences either (*P=0.91*). SJM167 and M9 contrast is close to be significant, indicating that it could be better classified as semi vigorous rather than as dwarfing. Besides, SJM167 was previously classified as semi invigorating when

grafted with Braeburn. Therefore, considering all the data, this rootstock could be classified as semi-vigorous.

No differences were observed when comparing root bark percentage in SJM189 with the controls, M9 and MM106 (*P=0.07* and *P=0.99* respectively). The high p-value in the contrast between SJM189 and MM106 indicates that these two rootstocks are very similar in root bark percentage. Nevertheless, the p-value in the contrast between M9 and SJM189, although is not statistically significant, is closer to be significant. In addition, SJM188 has the same dwarfing marker and the recombination must be taking place in the same area and it has been classified as semi invigoration. All these observations suggest that SJM189 could be in the semi vigorous group even though the statistical analysis is inconclusive.

Similarly, in SJP84-5174 contrasts with the reference rootstocks M9 and MM106, no statistically significant differences were detected (*P=0.2* and *P=0.97* respectively). Therefore, it is challenging to determine the dwarfing level of this rootstock based on the root bark data available.

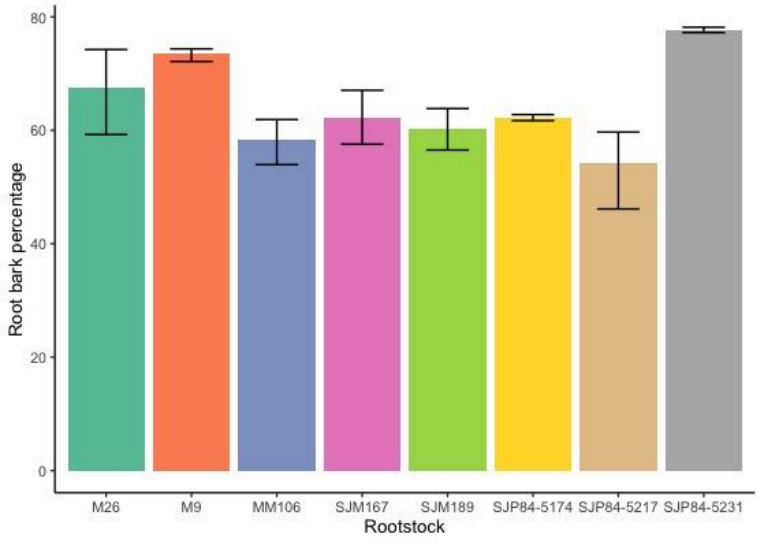

Figure 18. Effect of rootstock grafted with Gala on root bark percentage in trial D.

It is important to consider that there are many intermediate levels of dwarfing and it is complicated to classify a rootstock in one of these categories.

Canopy data from all of these recombinants was analysed by rootstock breeders at NIAB EMR as part of the breeding programme. The data was examined to see in which dwarfing level these genotypes were classified by breeders in terms of canopy volume and trunk diameter.

In summary, recombinant genotypes with markers MD11001 and MD11004 are semi invigorating according to the proportion of root bark. This indicates that these two markers are not needed to cause dwarfing since both are present in many individuals and the dwarfing phenotype is not expressed. On the other hand, M306-189 genotype was classified as dwarfing according to our root bark data. Canopy data confirmed that this genotype is certainly dwarfing. This indicates that only MD1103 marker is needed to cause dwarfing allowing us to narrow down the dwarfing region from 5.8Mb to 1.8Mb (Figure 19).

SJP84-5174 did not show significant differences when compared either to M9 or MM106. The p values when compared to MM106 were closer to 0.05 and closer to 1 when compared to M9, suggesting that this genotype is more similar to M9 than to MM106 and therefore can be classified as dwarfing or semi-dwarfing. This genotype was also categorised as semi dwarfing by breeders. This manifests that MD5001, MD5002 and MD5003 are not needed to cause dwarfing since they are not present in these genotypes and this is still somehow a bit dwarfing. In addition, we know from previous investigations (see previous report for details) that MD5007 and MD5004 are not in the dwarfing region since M9 and M27 are both dwarfing but they do not share these alleles, due to a recombination during the cross of M13 x M9 to generate M27. Consequently, the dwarfing QTL in chromosome 5 can be narrowed down to 2.1 Mb (Figure 19).

		M306-079		SJM167		SJM189		SJM188		M306-189		SJP84-5174	
Markers Chr5	Mbp	H1	H2	H1	H2	H1	H2	H1	H2	H1	H2	H1	H2
MD5001	41,4	129	133	119	132	119	132	119	132	128	132	130	128
MD5002	41,9	201	205	199	205	199	205	199	205	201	205	NA	NA
MD5003	42,2	140	142	176	141	176	141	176	141	139	141	141	150
MD5006	43,1	245	235	263	235	252	235	252	235	245	235	247	235
MD5007	45,2	NA	NA	262	276	272	276	272	276	NA	NA	264	276
MD5004	45,6	251	251	236	251	247	251	247	251	253	251	251	251
Markers Chr11	Mbp	H1	H2	H1	H2	H1	H2	H1	H2	H1	H2	H1	H2
MD11001	6,9	232	212	212	212	222	212	221	212	221	199	195	212
MD11004	7,5	210	203	205	203	191	191	191	191	198	205	NA	203
MD11005	8,3	98	98	116	116	122	116	122	116	98	98	NA	128
MD11002	9,8	141	141	146	155	159	155	159	155	140	147	140	142
MD11006	10,4	170	170	168	168	174	168	174	168	170	170	NA	164
MD11007	10,9	155	155	161	158	168	158	168	158	155	155	NA	159
MD11003	12,7	268	276	266	266	266	266	266	266	268	272	262	272

2,1 Mb

1,8 Mb

Non dwarfing

Dwarfing

Dwarfing

Figure 19. Summary of recombinant genotypes found in the breeding trials and its level of dwarfing based on the root bark data. Orange dashed lines delimiting the fine mapped root bark regions.

These findings are incredibly promising. The QTL regions have been notably narrowed down and accordingly, the number of genes located in these regions has been drastically reduced facilitating the identification of genes responsible for dwarfing.

Effect of scion on root bark percentage

The analysis of the breeding trial D revealed that both scion and rootstock have an effect on root bark percentage. However, the interaction was not significant although the p-value was very close to 0.05 (Table 9). Block was included in the analysis as a random effect.

Table 9. Type III Analysis of Variance Table with Satterthwaite's method showing the p values of the fixed effects.

Fixed effect	Sum sq	Mean sq	F value	Pr(>F)
Rootstock	1800.36	200.04	11.90	1.558e-08 ***
Scion	275.11	275.11	16.37	0.0002544 ***
Rootstock:Scion	199.00	39.8	2.36	0.0582147

M9, M26, MM106 and SJM167 rootstocks have higher root bark percentage when grafted with Gala. M9 rootstocks grafted with Braeburn have on average 64.7% of root bark percentage and those M9 grafted with Gala have a 73.5% of root bark. M26 rootstocks have 62% root bark percentage when grafted with Braeburn versus the 67.6% of root bark in rootstocks grafted with Gala. In MM106 rootstocks, the root bark was 51% and 58.3% when grafted with Braeburn and Gala respectively. The root bark percentage in SJM167 rootstock grafted with Gala was 10.9% higher than SJM167 rootstock grafted with Braeburn (62.3% and 51.4% respectively).

SJP84-5174 and SJP84-5217 rootstocks have a slightly lower root bark percentage when grafted with Gala. The remaining rootstocks are only grafted with one scion, therefore, contrast between them are not possible.

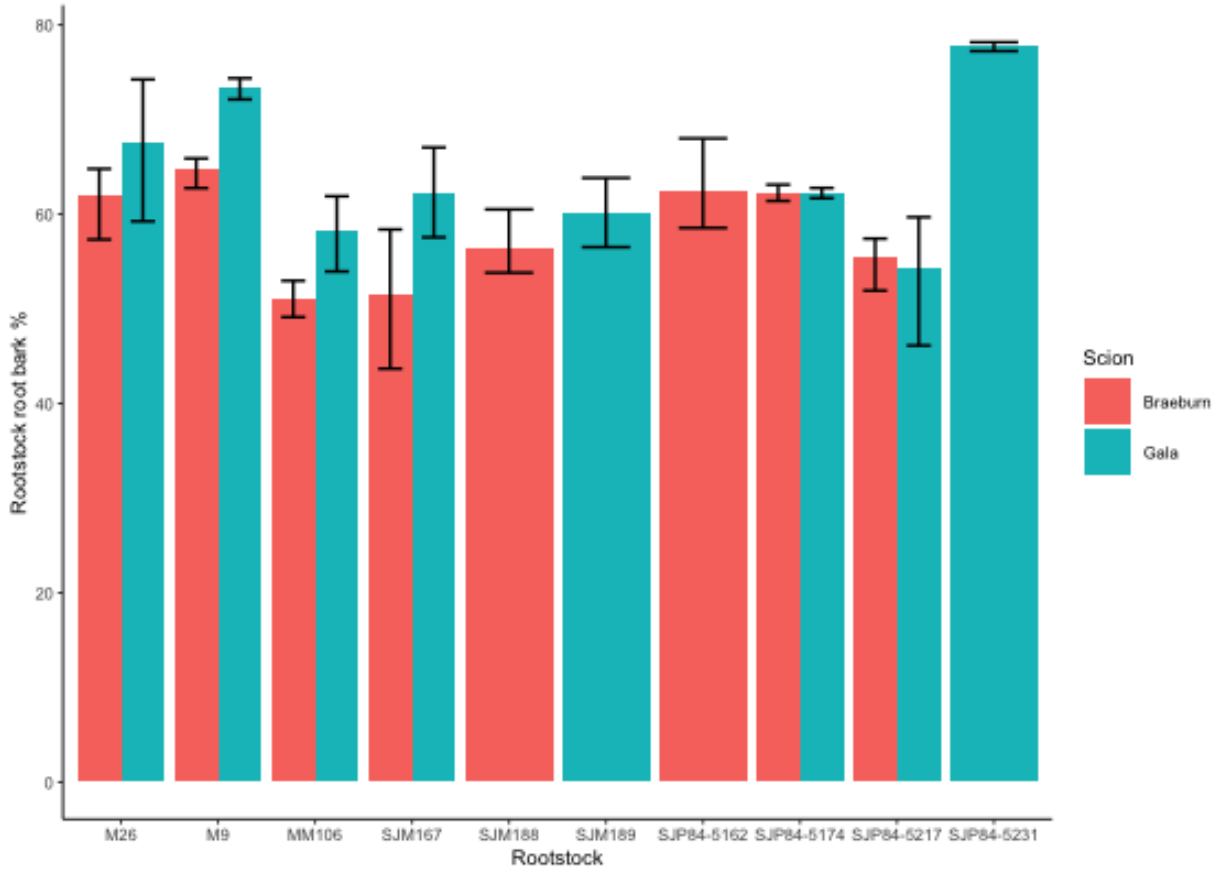

Figure 20. Effect of scion on rootstock root bark percentage in trial D.

An analogous analysis was done in Trial C, where rootstocks were also grafted with two scions, to determine if the effect of the scion was also influencing the root bark percentage in this trial. This analysis confirmed the previous finding, rootstock and scion have an effect on root bark percentage and in this analysis, also the interaction between rootstock and scion. Block was used as random effect (Table 10).

Table 10. Type III Analysis of Variance Table with Satterthwaite's method showing the p values of the fixed effects.

Fixed effect	Sum sq	Mean sq	F value	Pr(>F)
Rootstock	2943.37	420.48	18.52	2.268e-14 ***
Scion	571.48	571.48	25.17	3.159e-06 ***
Rootstock:Scion	594.86	84.98	3,74	0.001498 **

AR852-3, B24, M27, M26, M9 and R59 rootstocks have higher root bark percentage when grafted with Royal Gala. AR839-9 rootstocks have the same root bark percentage on average regardless of the scion. R104 rootstocks have a slightly higher root bark percentage when grafted with Braeburn.

The greatest difference in root bark percentage was identified in B24 rootstock with 45.6% of root bark grafted with Braeburn and 60.5% in rootstocks grafted with royal Gala. A considerable difference was also found in AR852-3 rootstocks, 53.1% root bark in Braeburn and 64% in those grafted with Royal Gala. M9 rootstocks also exhibited large differences between scions, 63.1% in Braeburn and 71.4% Braeburn. M27 and M26 rootstocks grafted with royal gala showed slightly higher root bark proportion than those grafted with Braeburn.

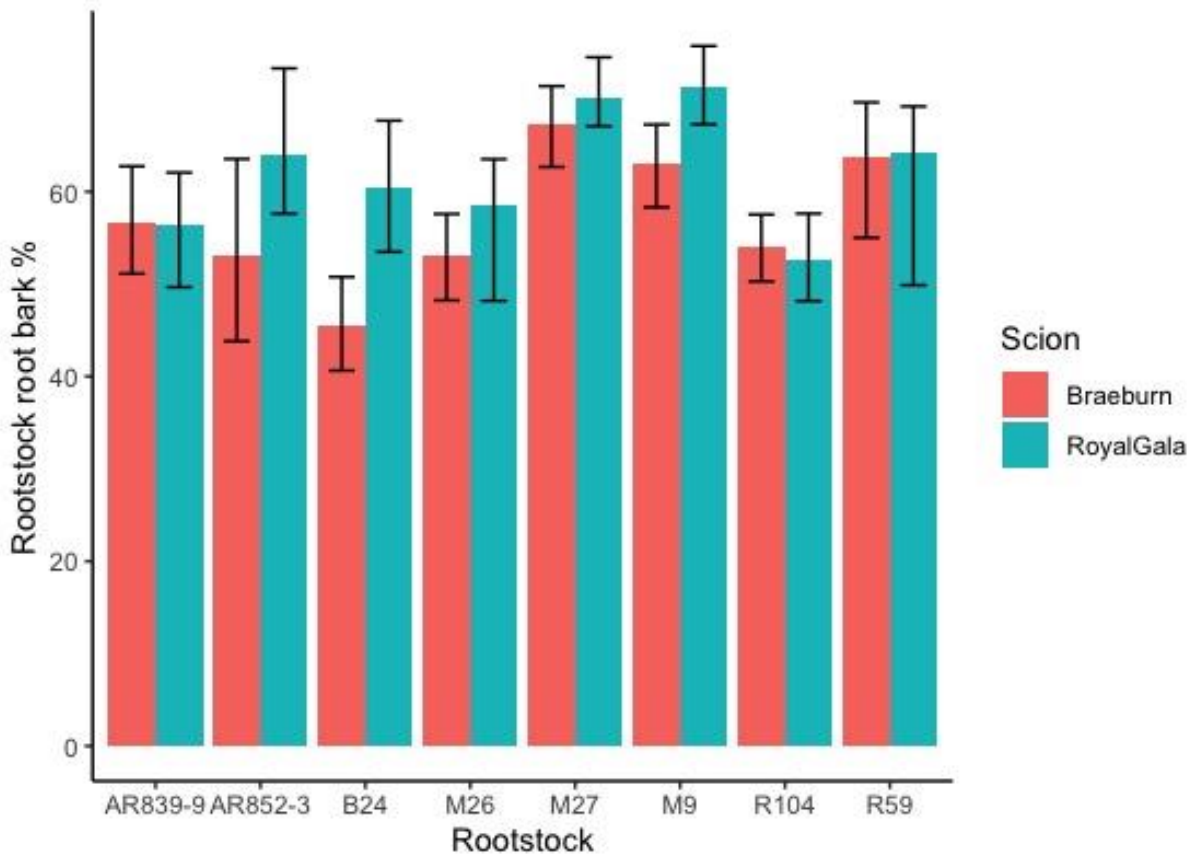

Figure 21. Effect of scion on rootstock root bark percentage in trial C.

The interaction between scion and rootstocks and its influence on root weight has been previously reported (Amos et al., 1930; Harrison et al., 2016a) but this the first time that we can see the effect of the scion in root bark percentage. This interaction between scion and rootstock is very interesting and this is another factor that breeders may have to consider.

Dwarfing markers will be screened in selected scions to determine which of the dwarfing markers are present and see if this can help to better understand this interaction.

Conclusions

No conclusions at this early stage of the project.

Knowledge and Technology Transfer

List of relevant presentations:

- AHDB PhD conference. Nottingham, 29th and 30th January 2020. Poster presentation. Special mention prize.
- CTP annual meeting. 4th August 2020. Project presentation.
- Genetic, genomic and breeding department meeting. 30th September 2020. Project presentation.

Glossary

ANOVA- analysis of variance

bp - base pairs

CK - cytokines

cM - centimorgan

FAA - formaldehyde alcohol acetic acid

FAOSTAT - food and agriculture organization of the united nations database

Mb - megabase pairs, millions of base pairs

PEG - polyethylene glycol

PCR - polymerase chain reaction

QTL - quantitative trait locus

RB - root bark

RSA - root system architecture

SL - strigolactones

SNP - single nucleotide polymorphism

SSR - single sequence repeat

References

Abreu, I., Poza, L., Bonilla, I. and Bolaños, L. 2014. Boron deficiency results in early repression of a cytokinin receptor gene and abnormal cell differentiation in the apical root meristem of Arabidopsis thaliana. Plant Physiology and Biochemistry 77, pp. 117–121.

Aloni, R., Aloni, E., Langhans, M. and Ullrich, C.I. 2006. Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. Annals of Botany 97(5), pp. 883–893.

Alvarez, J.M., Vidal, E.A. and Gutiérrez, R.A. 2012. Integration of local and systemic signalling pathways for plant N responses. Current Opinion in Plant Biology 15(2), pp. 185–191.

Amos, J., Hatton, R. G., Hoblyn, T. N. & Knight, R. C. 1930. The Effect of Scion on Root.: II. Stem-Worked Apples., Journal of Pomology and Horticultural Science, 8:3, 248-258.

Atkinson, C. 2001. Understanding how rootstocks dwarf fruit trees. Compact Fruit Tree 34: 46–49.

Beakbane, A.B. & Thompson, E.C., 1940. Anatomical Studies of Stems and Roots of Hardy Fruit Trees II. The Internal Structure of the Roots of Some Vigorous and Some Dwarfing Apple Rootstocks, and the Correlation of Structure with Vigour. Journal of Pomology and Horticultural Science, 17(2), pp.141–149.

Beakbane AB, Thompson EC. 1947. Anatomical studies of stem and roots of hardy fruit trees. IV. The root structure of some new clonal apple rootstocks budded with Cox's Orange Pippin. Journal of Pomology and Horticultural Science. 23, 203–226.

Bhosale, R., Giri, J., Pandey, B.K., Giehl, R.F.H., Hartmann, A., Traini, R., Truskina, J., Leftley, N., Hanlon, M., Swarup, K., Rashed, A., Voß, U., Alonso, J., Stepanova, A., Yun, J., Ljung, K., Brown, K.M., Lynch, J.P., Dolan, L., Vernoux, T. and Swarup, R. 2018. A mechanistic framework for auxin dependent Arabidopsis root hair elongation to low external phosphate. Nature Communications 9(1), p. 1409.

Bohn, M., Novais, J., Fonseca, R., Tuberosa, R. and Grift, T.E. 2006. Genetic evaluation of root complexity in maize. Acta Agronomica Hungarica 54(3), pp. 291–303.

Cummins JN, Aldwinckle HS (1983) Breeding apple rootstocks. In: Janick J (ed) Plant Breeding Reviews. Avi Publishing Co, Westport, Conn 294 – 394.

Di Guardo, M. et al., 2015. ASSIsT: an automatic SNP scoring tool for in- and outbreeding species. Bioinformatics, 31(23), pp.3873–3874.

Dobrzañski B., Rabcewicz J., and Rybczyñski R., 2006. Handling of Apple. IA PAS Press, Lublin, Poland.

Epstein, E. 2005. Mineral Nutrition of Plants: Principles and Perspectives, 2nd ed., Sinauer Associates, Sunderland, MA, USA. ISBN 0 87893 172 4.

Evans, R.C., Campbell, C.S., 2002. The origin of the apple subfamily (Maloideae; Rosaceae) is clarified by DNA sequence data from duplicated GBSSI genes. Am. J. Bot. 89, 1478–1484.

Fazio, G., Wan, Y., Kviklys, D., Romero, L., Adams, R., Strickland, D. and Robinson, T. 2014. Dw2, a New Dwarfing Locus in Apple Rootstocks and Its Relationship to Induction of Early Bearing in Apple Scions. Journal of the American Society for Horticultural Science, 39: 87–98.

Ferree, D.C., Clayton-Greene, K.A. and Bishop, B. 1993. Influence of orchard management system on canopy composition, light distribution, net photosynthesis and transpiration of apple trees. Journal of Horticultural Science 68(3), pp. 377–392.

Foster, T.M. et al., 2014. Key flowering genes including FT-like genes are upregulated in the vasculature of apple dwarfing rootstocks. Tree genetics & genomes, 10(1), pp.189–202.

Foster, T.M., Celton, J.-M., Chagné, D., Tustin, D.S. and Gardiner, S.E. 2015. Two quantitative trait loci, Dw1 and Dw2, are primarily responsible for rootstock-induced dwarfing in apple. Horticulture Research 2, p. 15001.

Gregory, P.J., Atkinson, C.J., Bengough, A.G., Else, M.A., Fernández-Fernández, F., Harrison, R.J. and Schmidt, S. 2013. Contributions of roots and rootstocks to sustainable, intensified crop production. Journal of Experimental Botany 64(5), pp. 1209–1222.

Harrison, N., Barber-Perez, N., Pennington, B., Cascant-Lopez, E. and Gregory, P.J. 2016a. Root system architecture in reciprocal grafts of apple rootstock-scion combinations. Acta horticulturae (1130), pp. 409–414.

Harrison, N., Harrison, R.J., Barber-Perez, N., Cascant-Lopez, E., Cobo-Medina, M., Lipska, M., Conde-Ruíz, R., Brain, P., Gregory, P.J. and Fernández-Fernández, F. 2016b. A new three-locus model for rootstock-induced dwarfing in apple revealed by genetic mapping of root bark percentage. Journal of Experimental Botany 67(6), pp. 1871–1881.

Hatton RG. ‘Paradise’ apple socks. J R Hort Soc 1917; 42: 361–399.

Heide, O.M. and Prestrud, A.K. 2005. Low temperature, but not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. *T*ree Physiology 25(1), pp. 109–114.

Jensen, P.J., Halbrendt, N., Fazio, G., Makalowska, I., Altman, N., Praul, C., Maximova, S.N., Ngugi, H.K., Crassweller, R.M., Travis, J.W. and McNellis, T.W. 2012. Rootstock-regulated gene expression patterns associated with fire blight resistance in apple. BMC Genomics 13, p. 9.

Jordan WR, Dugas WA Jr, Shouse PJ (1983) Strategies for crop improvement for drought-prone regions. Agr Water M 7:281–299

Keeney D.R. (1982) Nitrogen management for maximum efficiency and minimum pollution. In Nitrogen in Agricultural Soils, pp. 605–649. American Society of Agronomy, Madison, WI, USA.

Ljung, K., Hull, A.K., Celenza, J., Yamada, M., Estelle, M., Normanly, J. and Sandberg, G. 2005. Sites and regulation of auxin biosynthesis in Arabidopsis roots. The Plant Cell 17(4), pp. 1090–1104.

Ljung, K. 2013. Auxin metabolism and homeostasis during plant development. Development 140(5), pp. 943–950.

López-Bucio, J., Cruz-Ramírez, A. and Herrera-Estrella, L. 2003. The role of nutrient availability in regulating root architecture. Current Opinion in Plant Biology 6(3), pp. 280–287.

Ludlow, M.M. and Muchow, R.C. 1990. A Critical Evaluation of Traits for Improving Crop Yields in Water-Limited Environments. In: Advances in agronomy volume 43. Advances in Agronomy. Elsevier, pp. 107–153.

Lynch, J. 1995. Root architecture and plant productivity. Plant Physiology 109(1), pp. 7–13.

Lynch, J.P. and Brown, K.M. 2001. Topsoil foraging – an architectural adaptation of plants to low phosphorus availability. Plant and Soil (237): 225–237.

Lynch, J.P. 2007. Roots of the Second Green Revolution. Australian journal of botany 55(5), p. 493.

Ma, L., Hou, C.W., Zhang, X.Z., Li, H.L., De Guo Han, Wang, Y. and Han, Z.H. 2013. Seasonal Growth and Spatial Distribution of Apple Tree Roots on Different Rootstocks or Interstems. Journal of the American Society for Horticultural Science.

Marguerit, E., Brendel, O., Lebon, E., Van Leeuwen, C. and Ollat, N. 2012. Rootstock control of scion transpiration and its acclimation to water deficit are controlled by different genes. The New Phytologist 194(2), pp. 416–429.

Martín-Rejano, E.M., Camacho-Cristóbal, J.J., Herrera-Rodríguez, M.B., Rexach, J., Navarro-Gochicoa, M.T. and González-Fontes, A. 2011. Auxin and ethylene are involved in the responses of root system architecture to low boron supply in Arabidopsis seedlings. Physiologia Plantarum 142(2), pp. 170–178.

Näsholm, T., Kielland, K. and Ganeteg, U. 2009. Uptake of organic nitrogen by plants. The New Phytologist 182(1), pp. 31–48.

Noiton D.A.M., Alspach P.A. (1996): Founding clones, inbreeding, coancestry, and status number of modern apple cultivars. Journal of the American Society for Horticultural Science, 121: 773–782.

Norelli, J.L., Holleran, H.T., Johnson, W.C., Robinson, T.L. and Aldwinckle, H.S. 2003. Resistance of Geneva and Other Apple Rootstocks to *Erwinia amylovora*. Plant disease 87(1), pp. 26–32.

Osmont, K.S., Sibout, R. and Hardtke, C.S. 2007. Hidden branches: developments in root system architecture. Annual review of plant biology 58, pp. 93–113.

Paez-Garcia, A., Motes, C.M., Scheible, W.-R., Chen, R., Blancaflor, E.B. and Monteros, M.J. 2015. Root traits and phenotyping strategies for plant improvement. Plants 4(2), pp. 334–355.

Petersson, S.V., Johansson, A.I., Kowalczyk, M., Makoveychuk, A., Wang, J.Y., Moritz, T., Grebe, M., Benfey, P.N., Sandberg, G. and Ljung, K. 2009. An auxin gradient and maximum in the Arabidopsis root apex shown by high-resolution cell-specific analysis of IAA distribution and synthesis. *T*he Plant Cell 21(6), pp. 1659–1668.

Pilcher, R.L.R., Celton, J.M., Gardiner, S.E. and Tustin, D.S. 2008. Genetic Markers Linked to the Dwarfing Trait of Apple Rootstock 'Malling 9.' Journal of the American Society for Horticultural Science. 113: 100-106.

Preston, A.P. 1955. Apple rootstock studies: Malling Merton rootstocks. J. Hort. Sci. 30:25-33.

Rasmussen, A., Depuydt, S., Goormachtig, S. and Geelen, D. 2013. Strigolactones fine-tune the root system. Planta 238(4), pp. 615–626.

Robinson, T.L. 2007. Effects of tree density and tree shape on apple orchard performance. Acta horticulturae (732), pp. 405–414.

Schindelin, J.; Arganda-Carreras, I. & Frise, E. et al. (2012), "Fiji: an open-source platform for biological-image analysis", *Nature methods* 9(7): 676-682.

Seleznyova, A.N., Thorp, T.G., White, M., Tustin, S. and Costes, E. 2003. Application of architectural analysis and AMAPmod methodology to study dwarfing phenomenon: the

branch structure of “Royal Gala” apple grafted on dwarfing and non-dwarfing rootstock/interstock combinations. *A*nnals of Botany 91(6), pp. 665–672.

Sultan, S.E. 2003. Phenotypic plasticity in plants: a case study in ecological development. Evolution & Development 5(1), pp. 25–33.

Strock, C.F., Morrow de la Riva, L. and Lynch, J.P. 2018. Reduction in root secondary growth as a strategy for phosphorus acquisition. Plant Physiology 176(1), pp. 691–703.

Takano, J., Wada, M., Ludewig, U., Schaaf, G., von Wirén, N. and Fujiwara, T. 2006. The Arabidopsis major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. The Plant Cell 18(6), pp. 1498–1509.

Tamura, F. 2012. Recent advances in research on japanese pear rootstocks. *J*ournal of the Japanese Society for Horticultural Science 81(1), pp. 1–10.

Tegeder, M. and Rentsch, D. 2010. Uptake and partitioning of amino acids and peptides. Molecular Plant 3(6), pp. 997–1011.

Untergasser, A. et al., 2012. Primer3--new capabilities and interfaces. Nucleic Acids Research, 40(15), p.e115.

Vidal, E.A., Araus, V., Lu, C., Parry, G., Green, P.J., Coruzzi, G.M. and Gutiérrez, R.A. 2010. Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in Arabidopsis thaliana. Proceedings of the National Academy of Sciences of the United States of America 107(9), pp. 4477–4482.

Vitousek, P.M., Mooney, H.A., Jubchenco, J. and Melillo, J.M. 1997. Human domination of Earth’s ecosystems. Science 277, pp. 494–499.

Wang, X., Wu, P., Hu, B. and Chen, Q. 2002. Effects of nitrate on the growth of lateral root and nitrogen absorption in rice. Acta Botanica Sinica 44: 678 – 683.

Webster, A.D., 1995. Rootstock and interstock effects on deciduous fruit tree vigour, precocity, and yield productivity. New Zealand journal of crop and horticultural science, 23(4), pp.373–382.

Webster, T., Tobutt, K. and Evans, K. 2000. Breeding and Evaluation of New Rootstocks for Apple, Pear and Sweet Cherry. In: International dwarf fruit association.

Weinbaum, S.A., Johnson, R.S. and DeJong, T.M. 1992. Causes and Consequences of Overfertilization in Orchards. HortTechnology 2:112-120.

Werner, T., Motyka, V., Strnad, M. and Schmülling, T. 2001. Regulation of plant growth by cytokinin. Proceedings of the National Academy of Sciences of the United States of America 98(18), pp. 10487–10492.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H. and Schmülling, T. 2003. Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. The Plant Cell 15(11), pp. 2532–2550.

White, P.J., George, T.S., Gregory, P.J., Bengough, A.G., Hallett, P.D. and McKenzie, B.M. 2013. Matching roots to their environment. Annals of Botany 112(2), pp. 207–222.

Xu, G., Fan, X. and Miller, A.J. 2012. Plant nitrogen assimilation and use efficiency. Annual review of plant biology 63, pp. 153–182.

Zheng, C., Shen, F., Wang, Y. *et al.* Intricate genetic variation networks control the adventitious root growth angle in apple. *BMC Genomics* **21,** 852 (2020).

