Comparison of fire blight resistance screening methodologies

M.B. Horner¹, E.G. Hough¹, D.I. Hedderley², N.M. How¹ and V.G.M. Bus¹

¹The New Zealand Institute for Plant & Food Research Limited, Private Bag 1401, Havelock North 4157, New Zealand

²Plant & Food Research, Private Bag 11600, Manawatu Mail Centre, Palmerston North, 4442, New Zealand

Corresponding author: mary.horner@plantandfood.co.nz

Abstract Fire blight, a bacterial disease caused by *Erwinia amylovora*, is an ongoing problem for pipfruit growers, with few control options available. Most commercial cultivars and rootstocks are highly susceptible to the disease. Breeding of fire blight-resistant scions and rootstocks to manage the disease is a major goal of the New Zealand apple breeding programme. The main mode of disease establishment is through flowers. However, the breeding programme currently evaluates disease resistance through shoot inoculations. This study compared the degree of resistance in 109 progeny from a ‘Royal Gala’ × *Malus robusta* ‘Robusta 5’ family assessed by shoot inoculation and by floral inoculations. Results indicate that the two measures of resistance do not correlate well, and that different quantitative trait loci may be involved in flower and shoot resistance. Management of fire blight through the implementation of resistant cultivars will require resistance screening on both shoot and flower assessments.

Keywords *Erwinia amylovora*, resistance breeding, floral inoculation, shoot inoculation.

INTRODUCTION

Fire blight has been an ongoing issue for apple growers in New Zealand since it was first reported by Cockayne (1920). The presence of fire blight infection in orchards poses both production and market access problems. The disease is caused by the bacterial pathogen *Erwinia amylovora* (Burrill). Disease severity varies with cultivar and from year to year, depending on the prevailing weather conditions. The pathogen can be very destructive as it can invade blossoms, fruits, vegetative shoots, woody tissues and rootstock crowns (Hildebrand 1937; Thomson 2000). *Erwinia amylovora* invades the host predominantly via susceptible flowers through natural openings. It has been reported

to grow epiphytically on the stigma and infect via the nectarthodes (Hildebrand 1937; Thomson 2000). Blossom infections lead to a reduction in the current season crop yield, while the following year’s yield will be reduced if the fruit spurs or the annual wood that bears the following season’s fruit spurs are also killed (Dye 1949).

Fire blight is difficult to manage, as most commercial cultivars and rootstocks are highly susceptible to the disease (Van der Zwet & Keil 1979) and there are very few chemical control options. Resistance to fire blight in *Malus* ranges from highly susceptible to highly resistant. Many apple cultivars have been evaluated for their resistance against fire blight by controlled

inoculation of plant material (van der Zwet et al. 2011). Adoption of less susceptible cultivars offers the possibility of improved fire blight control, and hence breeding of fire blight-resistant scions and rootstocks is a major goal of the New Zealand apple breeding programme.

The resistance breeding programme currently evaluates disease resistance by shoot inoculation screening. However, the main mode of fire blight establishment is through floral infection. This research evaluated whether there is any correlation between observed fire blight resistance when the same plants from a 'Royal Gala' x *Malus robusta* 'Robusta 5' family were phenotyped for fire blight resistance by artificial inoculation onto either shoots or flowers.

MATERIALS AND METHODS

Plant material

The fire blight susceptible cultivar 'Royal Gala' (RG) of apple *Malus* × *domestica* was crossed in 1998 with the fire blight-resistant species accession *Malus robusta* 5 (R5). A single tree each of the resulting progeny was grafted onto 'Malling 27' rootstocks and grown in the Plant & Food Research orchard, Havelock North, New Zealand. One-hundred-and-nine of these trees were selected for the trial, ensuring a range of susceptibilities to *E. amylovora* as determined in previous assessments. These orchard trees themselves were used in the floral inoculation assessments and had not been exposed to *E. amylovora* prior to this experiment.

One-year-old wood from each of the 109 different flowering progeny was grafted onto potted 'Malling 9' rootstocks and grown as a single shoot. These trees (10 replicates of each) were used in shoot inoculations. Four weeks before inoculation, the potted trees were transferred from outside into a glasshouse and grown at 25–27°C (day) and 20°C (night) at 80% humidity until 1 week before inoculation, when relative humidity was increased to 95%.

Inoculum production

The *E. amylovora* isolate 236 (isolated from *Malus domestica* in 1998, Blenheim, New Zealand) from the Plant & Food Research culture collection was used in these experiments. This isolate was

chosen based on its high virulence and frequency of successful infection. Inoculum was prepared by growing the bacteria on a King's B agar (King et al. 1954) at 26°C. After 48 h, bacteria were harvested and suspended at a concentration of 1×10^9 cfu/ml in a phosphate buffered saline solution pH 7.2. Bacterial concentration was determined by a spectrophotometer (Pye Unicam SP6 Series, Cambridge, England) and then verified by colony counts on dilution plates.

Phenotyping for shoot resistance

Actively growing shoots (minimum length of 16 cm) from each of the 109 progeny (up to 10 replicates of each) were inoculated in 2012 and 2013 by cutting the tips of the two uppermost unfolded leaves with scissors that had been dipped in a 10^9 cfu/ml suspension of *E. amylovora*. The inoculated plants were kept in the glasshouse for a further 4 weeks, after which the length of necrosis of each shoot and total shoot length were measured.

Phenotyping for floral resistance

In 2009, ten floral clusters on each of the 109 flowering trees in the orchard were tagged, then individually spray inoculated with 180 µl of an *E. amylovora* suspension at 1×10^6 cfu/µl. Floral clusters contained a king flower, which was just opening or less than 1 day old. Inoculations took place between 2 and 30 September 2009 as flower clusters reached the standard cluster maturity. There was little or no risk of natural fire blight infection during the inoculation period based upon the Cougar blight model in metwatch (<http://www.hortplus.metwatch.co.nz>). Bacterial inoculum (180 µl) was sprayed onto floral clusters using a DeVilbiss® atomiser model No. 15. Individual floral clusters were bagged (plastic) and then covered with aluminium foil immediately after inoculation to minimise solar heating inside the bag. Bags were removed 3 days later.

Floral clusters were assessed for fire blight infection at 9–12 days post-inoculation and again at 27–37 days post-inoculation. Individual floral clusters were given a disease severity rating based on the presence of necrosis and how far the disease had travelled. Disease severity ratings were: 0 = no infection, 1 = floral infection,

2= infection in flowers and peduncle, 3= floral and bourse infection, 4= floral, bourse and bourse-shoot infection, 5= infection travelled up to 4 cm along branch, 6= infection travelled greater than 4 cm along branch. Disease resistance scores were based on the disease severity ratings where a disease severity rating of 0-1 was highly resistant and a severity rating of 5-6 was highly susceptible.

Statistical analysis

Data were analysed using Microsoft Excel and Genstat (version 14, 2011, VSNi Ltd, Hemel Hempstead, UK). A mean length of necrotic shoot was estimated for each plant using Genstat's CENSOR procedure to account for the possibility that infections which grew the whole length of the shoot would have grown further on a longer shoot. The length of necrotic shoot tissue relative to total shoot length of the shoot was also calculated. The mean shoot resistance score was the percentage of stem necrosis where 0 was highly resistant and 100 was highly susceptible. Progeny were also classified into two groups by whether any of the replicates became infected ('Some') or not ('None'). This was done for both the 2012 and 2013 data, creating a 2×2 classification ('Some' or 'None' in 2012 × 'Some' or 'None' in 2013). Distribution of floral mean severity scores was compared between progeny that had no shoot infection in either 2012 or 2013, and progeny that had shoot infections in both 2012 and 2013 using the two-sample Kolmogorov-Smirnov test.

Floral disease resistance was summarised in several ways: arithmetic means of the severity ratings, the proportion of the ten floral clusters that were scored 3 or higher (i.e. infection had spread beyond the flower), and the proportion of the ten flowers that were scored 5 or higher (i.e. infection had spread into wood).

Correlation between the fire blight shoot resistance score (percentage shoot necrosis) and the floral resistance score was determined using Pearson's correlation.

RESULTS

Shoot resistance assessment

The mean percentage shoot necrosis for the various progeny ranged from 0-73%. Analysis of the results indicated that the percentage of stem necrosis was

not consistent from year to year. The correlation between the mean shoot necrosis lengths in the two years was 0.474, significant but not strong. Of the 88 progeny tested in both years, 16 had some infection in both years; 21 had infections in one year but not the other, and 51 were not infected in either year. A chi-squared test of association between the 'None'/'Some' classification in 2012 and 2013 was significant ($\chi^2=20.0$, 1 df, $P<0.001$), suggesting some progeny are consistently uninfected and some consistently infected.

Flower resistance assessment

There was a wide range of resistance levels in the RG×R5 population when screened through artificial floral inoculations (Figure 1). Resistance scores ranged from 0 to 6, where 0 was highly resistant and 6 highly susceptible. Inspection of the floral results, taking into account the different inoculation dates in case variations in the strength/viability of the inoculum affected the results, revealed that the different inoculation dates did not influence the patterns of the resistance score, as all dates showed similar resistance patterns. The correlation between the mean scores of resistance 9-12 days post inoculation (assessment 1) and 27-37 days post-inoculation (assessment 2) was 0.598.

Comparison of shoot inoculation scores with floral inoculation scores

When the floral resistance score was compared with the shoot resistance score (Figure 2), there was no significant correlation between the two measures (correlations ranged from 0.075 to -0.133). In many cases, progeny with a zero mean percent shoot necrosis (resistant) had a wide range of floral scores from a ranking of zero to five. Similarly, progeny that had a high floral resistance score (0-1) showed a variable percentage shoot necrosis resistance ranking, which ranged from 0 to 65% shoot necrosis (Figure 2). The low level of correlation remained the same whether the 2012 or 2013 shoot assay data or the first or second floral assessments data were used. The results were checked for any difference between inoculation dates for the floral assay in case variations in the strength or viability of the inoculum affected the results. All the dates showed a similar result of no correlation.

Figure 1 Mean resistance scores at 9 to 12 and 27 to 37 days post inoculation (DPI) for progeny derived from a 'Royal Gala' × 'Robusta 5' cross when screened by artificial floral inoculation. 0= no infection, 1= floral infection, 2= infection in flowers and peduncle, 3= floral and bourse infection, 4= floral, bourse and bourse shoot infection, 5= infection travelled up to 4 cm along branch, 6= infection travelled greater than 4 cm along branch.

Figure 2 Correlation between the mean floral resistance score at 27-31 days post inoculation (0= no infection, 1= floral infection, 2= infection in flowers and peduncle, 3= floral and bourse infection, 4= floral, bourse and bourse shoot infection, 5= infection travelled up to 4 cm along branch, 6= infection travelled greater than 4 cm along branch) and shoot resistance score (measured as mean percentage shoot necrosis) when progeny from a 'Royal Gala' × 'Robusta 5' cross were screened for fire blight resistance by shoot and floral inoculation screening.

Using the two-by-two classification of either 'None' or 'Some' shoot infection in 2012 and 'None' or 'Some' shoot infection in the 2013 shoot assays for individual progeny, the distribution of floral mean scores was similar for progeny that never showed shoot infection and progeny that had shoot infection in both years (Kolmogorov Smirnov 2 sample test; chi-square = 0.54 on 2 df, $P=0.764$ for assessment 1; chi-square = 0.63 on 2 df, $P=0.729$ for assessment 2). The proportion of flowers where the infection had spread beyond the flower (scores 3-6) was similar regardless of which of the four shoot classifications the progeny was. The same was true for the proportion of flowers where infection had spread into wood (scores 5-6), and the proportion of flowers showing no infection (chi-squared tests, P values ranged from 0.573 and 0.989).

DISCUSSION

This comparison of observed fire blight resistance when screened through flowers or shoots indicated that there was no correlation between the two resistance modes in the progeny derived from a RG×R5 cross. This strongly suggests that the major quantitative trait loci (QTL) for shoot resistance on linkage group 3 (LG3) of R5 is not involved in flower resistance, but, for example, the LG7 QTL may be (Gardiner et al. 2012). This result is similar to the findings of Thibault & Le Lezec (1990), who showed that the correlation between susceptibility in shoots and of flowers in 'Gala' apple trees was weak ($0.25 < r < 0.44$). Currently, shoot screening remains the main method of fire blight resistance screening in apple tree breeding. This method is based on the calculation of the percentage necrosis of the shoot rather than the necrosis length *per se* to accommodate for shoot length variation. However, since one of the main modes of infection is through flowers (van der Zwet & Keil 1979), screening through the flowers, at some stage in the breeding programme, is warranted. This will complement the shoot resistance measurements, and together they will contribute to the long-term goal of durable fire blight resistance on a whole tree basis.

In general, resistance to *E. amylovora* is quantitative, and several QTLs for resistance

have been identified in apple by genetic analysis, explaining from well below 10% (Calenge et al. 2005; Le Roux et al. 2010; Durel et al. 2009; Khan et al. 2012) to 65-83% of the phenotypic variation (Gardiner et al. 2012; Parravicini et al. 2011; Peil et al. 2007; Emeriewen et al. 2014). However, these results have been based upon data where the phenotyping of populations has been carried out by artificial inoculations of shoots and not floral inoculations. Results of the current work indicate that there are probably different quantitative trait loci involved in flower and shoot resistance. Further work will be performed to identify QTLs associated with the observed floral resistance identified in the RG×R5 family.

ACKNOWLEDGEMENTS

This work was part of the Low Impact Disease Control programme funded by the Foundation for Research, Science and Technology (Contract C06X0810).

REFERENCES

- Calenge F, Drouet D, Denancé C, van de Weg WE, Brisset MN, Paulin JP, Durel CE 2005. Identification of a major QTL together with several minor additive or epistatic QTLs for resistance to fire blight in apple in two related progenies. *Theoretical and Applied Genetics* 111: 128–135.
- Cockayne AH 1920. Fire blight, a serious disease of fruit trees. *New Zealand Journal of Agriculture* 20: 156–157.
- Durel C-E, Denance C, Brisset MN 2009. Two distinct major QTL for resistance to fire blight co-localize on linkage group 12 in apple genotypes 'Evereste' and *Malus floribunda* clone 821. *Genome* 52: 139–147.
- Dye DW 1949. 23rd Annual Report. Department of Scientific and Industrial Research, New Zealand. 88 p.
- Emeriewen O, Richter K, Kilian A, Zini E, Hanke M-V, Malnoy M, Peil A 2014. Identification of a major quantitative trait locus for fire blight resistance in the wild apple species *Malus fusca*. *Molecular Breeding* doi: 10.1007/s11032-014-0043-1.

- Gardiner SE, Norelli JL, de Silva N, Fazio G, Peil A, Malnoy M, Horner M, Bowatte D, Carlisle C, Wiedow C, Wan Y, Bassett CL, Baldo AM, Celton J-M, Richter K, Aldwinckle H, Bus VGM 2012. Candidate gene markers associated with quantitative trait loci for fire blight resistance in *Malus* “robusta 5” accessions. *BMC Genetics* 13(1): 25.
- Hildebrand EM 1937. Infectivity of the fire-blight organism. *Phytopathology* 27: 850–852.
- Khan MA, Zhao YF, Korban SS 2012. Molecular mechanisms of pathogenesis and resistance to the bacterial pathogen *Erwinia amylovora*, causal agent of fire blight disease in Rosaceae. *Plant Molecular Biology Reporter* 30: 247–260.
- King EO, Ward, MK, Raney DE 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *Journal of Laboratory and Clinical Medicine* 44: 301–307.
- Le Roux PMF, Khan MA, Broggin GAL, Duffy B, Gessler C, Patocchi A 2010. Mapping of quantitative trait loci for fire blight resistance in the apple cultivars ‘Florina’ and ‘Nova Easygro’. *Genome* 53: 710–722.
- Parravicini G, Gessler C, Denancé C, Lasserre-Zuber P, Vergne E, Brisset M, Patocchi A, Durel C, Broggin GAL 2011. Identification of serine/threonine kinase and nucleotide-binding site–leucine-rich repeat (NBS-LRR) genes in the fire blight resistance quantitative trait locus of apple cultivar ‘Evereste’. *Molecular Plant Pathology* 12: 493–505.
- Peil A, Garcia-Libreros T, Richter K, Trognitz FC, Trognitz B, Hanke MV, Flachowsky H 2007. Strong evidence for a fire blight resistance gene of *Malus robusta* located on linkage group 3. *Plant Breeding* 126: 470–475.
- Thibault B, Le Leze M 1990. Sensibilité au feu bactérien des principales variétés de pommier et du Poirier utilisées en Europe. In: *Fire Blight of Pomoideae (Erwinia amylovora*, Burrill, Winslow et al.). Applied Research in Europe (1978–1988), EUR 12601, ECSC-EEC-EAEC, Brussels-Luxembourg. Pp. 96–109.
- Thomson SV 2000. Epidemiology of fire blight. In: Vanneste JL ed. *Fire Blight: The Disease and its Causative Agent, Erwinia amylovora*. CABI Publishing, CAB International, Wallingford, UK. Pp. 9–36.
- van der Zwet T, Keil HL 1979. Fire blight—A bacterial disease of rosaceous plants. United States Department of Agriculture Handbook 510. 200 p.
- van der Zwet T, Orolaza-Halbrendt N, Zeller W 2011. *Fire Blight: History, Biology and Management*. APS Press, St Paul, MN, USA. 420 p.