Population differentiation of the apple scab fungus Venturia inaequalis on cultivars within a mixed cultivar orchard

POPULATION DIFFERENCE OF THE APPLE SCAB FUNGUS *VENTURIA INAEQUALIS* ON CULTIVARS WITHIN A MIXED CULTIVAR ORCHARD

T.A.J. PASSEY[1,2], M.W. SHAW[2] and X.-M. XU[1]
[1]NIAB EMR, East Malling, Kent, ME19 6BJ, United Kingdom
[2]University of Reading, School of Agriculture, Policy and Development, Reading, RG6 6AR, United Kingdom
Corresponding author E-mail: Tom.Passey@emr.ac.uk

SUMMARY

Apple cultivars differ in their resistance to the fungal pathogen *Venturia inaequalis*, the causal agent of apple scab. Mixed cultivar orchards, where the cultivars present have differing resistance to *V. inaequalis,* have been shown to reduce the levels of scab compared to monoculture. To maximise the mixture effect of reducing scab development, cultivars need to be selected with maximum differences in their scab resistance. One indirect, yet efficient, method of selecting such cultivars is to quantify population differences of scab from different cultivars, which are expected to largely reflect the differences in the resistance to the pathogen.

We sampled early season scab lesions from different cultivars in two mixed cider cultivar orchards in the Southwest of England and from various desert and cider cultivars in an orchard in the Southeast of England. Using Simple Sequence Repeat (SSR) markers we compared the scab populations sampled from the different cultivars.

Scab populations from different cultivars differed significantly, depending on specific pairs of cultivars; however, larger differences appear to be among fungal populations from different sites. The results demonstrate that certain cultivars likely share much of their genetic resistance factors to *V. inaequalis*. For dessert apple the scab populations on Cox, Gala, Bramley and Fiesta were not different and therefore it is not advisable to plant these cultivars in the same orchard with a view to reduce scab development. On the other hand, a reduction in scab would be more likely if one of the above cultivars is planted together with Golden Delicious, Red Falstaff or Spartan.

There was a very large difference in the scab population from cv. Three Counties and populations from all other cider cultivars. This was particularly surprising given the shared parentage (Dabinett x James Grieve) between Three Counties and all but one of the other cider cultivars sampled, suggesting considerable differences in the resistance to *V. inaequalis* between the two parents. It also indicates that selection of cultivars for inclusion in mixed orchards cannot be reliably made based on pedigree information alone.

Key words: apple scab, cultivar mixtures, population comparison, resistance

INTRODUCTION

Apple scab is one of the most important diseases economically to the apple industry worldwide. Caused by the ascomycete *Venturia inaequalis*, annual epidemics lead to large losses of marketable fruit due to the unsightly lesions not accepted by retailers and consumers. Left uncontrolled the disease can lead to poor tree health and reduced yields. Current control is predominantly through a programme of fungicide sprays aided by forecasting models (Beresford and Manktelow, 1994; Berrie and Xu, 2003). Emergence of resistance to fungicides from the pathogen, the demand from consumers and policy makers to reduce levels of pesticide use and the cost of chemical control of scab make the need for alternatives a necessity.

The natural resistance of a host is often used to control disease development. The only R-gene that has been bred into new commercial cultivars of apple (*Malus* x *domestica)* is the Rvi6 (Vf) gene from *M. Floribunda.* However this resistance has been overcome (Parisi *et al.*, 1993;

Roberts and Crute, 1994) and is only an option for newly bred cultivars not for popular current cultivars. However it is known that scab isolates from one cultivar cannot necessarily infect another cultivar (Sierotzki *et al.*, 1994; Koch *et al.*, 2000; Barbara *et al.*, 2008). This indicates that susceptible apple cultivars may have differing resistance factors to certain strains of scab. Incorporating cultivars in an orchard with these differences in resistance factors can reduce the incidence of scab. Mixing cultivars to reduce levels of scab has been shown to work in an orchard with a susceptible cultivar and a cultivar carrying the Rvi6 R-gene reducing leaf scab incidence in the range of 7.3 to 22.5% (depending on year and orchard design) compared to monoculture, improved further when a spray programme was applied (14.8 to 75.1% reduction) (Didelot *et al*. 2007). Similarly, this can lead to reduced incidence of fruit scab, further improved when coupled with sanitation (Parisi *et al*. 2013).

The design of a mixed orchard is important to achieve disease reduction compared to monoculture. Mixing within rows is more effective than alternate rows of cultivars, which in turn is more effective than blocks (Blaise and Gessler, 1994; Didelot *et al.*, 2007). This is due to the Genotype Unit Area (GUA) where the larger the area of host with the same genotype the smaller the disease reduction (Mundt, 2002). Therefore the closer the trees of the same cultivar are together the larger the GUA and the higher chance of autoinfection. Within row mixtures are not likely to be introduced into a commercial setting as increased management costs would be too high compared to other types of mixture, let alone monoculture.

One necessary requirement for using mixture to manage diseases is that the cultivars to be used in a mixture must differ in their resistance factors to the target pathogen(s). In this study we compared scab populations on different cultivars within an orchard and used that knowledge to infer which cultivars have differential resistance to scab (hence can be used together in a mixture orchard). Fungal population differences were determined at the molecular level (using Simple Sequence Repeat (SSR) markers). Leaf scab lesions were sampled from several popular desert cultivars and cider cultivars.

MATERIALS AND METHODS

Sampling

Orchard WM132 at NIAB EMR (Kent, UK) has blocks of three rows of *Malus* x *domestica* cv. Cox and three rows of *Malus* x *domestica* cv. Fiesta separated by a block of three rows of *Malus* x *domestica* cv. Royal Gala (Gala); each row has 12 trees. This orchard (ca. 15 years old) has had no fungicide programmes applied since planting, but has received minimal orchard husbandry (pruning, mowing). Scabbed leaves were sampled from the trees in late spring 2012. In 2013 potted trees of each of the three cultivars were placed in the orchard within their own cultivar. At the same time, potted trees of *Malus* x *domestica* cv. Bramley, *Malus* x *domestica* cv. Golden Delicious, *Malus* x *domestica* cv. Red Falstaff, *Malus* x *domestica* cv. Rosette and *Malus* x *domestica* cv. Spartan were placed in this orchard, two trees within each of Cox and Gala orchard trees and one within Fiesta. These potted trees were put in the same orchard again in 2014, this time one tree in each of Cox and Gala, and three within Fiesta. In addition, two potted trees of each of three cider cultivars, *Malus* x *domestica* cv. Angela, *Malus* x *domestica* cv. Dabinett and *Malus* x *domestica* cv. Somerset Redstreak were placed within each of the three cultivars in 2014 (six trees in total). All potted trees used were placed into the orchard at bud burst (approximately 1 m from the orchard tree). They were moved back to a polytunnel after 3-4 weeks and lesions sampled from these trees after a further three weeks.

Two commercially managed cider orchards in Southwest England were also sampled. First, an experimental orchard planted in 2008 in Staunton-on-Wye, Herefordshire was sampled at the end of spring 2012. This orchard was designed as a complete randomised block. The orchard contained the cultivars Angela, Dabinett, *Malus* x *domestica* cv. Katja (known as Katy in the UK and in this study), *Malus* x *domestica* cv. Lizzy and *Malus* x *domestica* cv. Tina on six different rootstock/interstock combinations. We sampled scab lesions from two plots (two different rootstocks) of each cultivar within one block, where all trees sampled were all in an area approx. 12 m x 70 m. Second, St Monica's Orchard, planted in 2009 in Sandford, Somerset, was sampled at the end of spring 2014. This is a mixed orchard of 24 new cider cultivars (colloquially known as "The Girls"), one row (approx. 120 m) of each between rows of Katy. Seven of the 24 cultivars had been identified in a previous scab survey as susceptible to scab, however only *Malus* x *domestica* cv. Gilly, *Malus* x *domestica* cv. Three Counties, Tina and *Malus* x *domestica* cv. Vicky had enough scab to sample in 2014. Infected leaves from Katy were also sampled.

For sampling of all sites, leaves with freshly sporulating, discrete scab lesions, were selected and placed into paper bags, no more than one leaf per shoot. For all leaves collected, a single discrete lesion per leaf was cut out with a 5 mm cork borer, placed in a 2 ml micro tube, left to air dry at room temperature, 2x 4 mm ball bearings added to the tube and then transferred to a -20 °C freezer.

DNA extraction and screening

DNA was extracted directly from the lesion on the leaf disc. The leaf disc was disrupted in a MM2 oscillating mill (Retsch, Haan, Germany) and DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions with all optional steps. DNA was eluted with 100 µl elution buffer into a 1.5ml micro tube. DNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA) and stored at -20 °C.

The SSR primers used (Table 1), PCR and thermal cycle conditions, as well as the procedure for genotyping followed published protocols (Passey *et al.* 2016).

Table 1 Simple Sequence Repeat primer pairs (5' to 3') used for screening apple scab isolates and the range of the alleles in present study

SSR	Fluorescent label-Forward primer	Reverse Primer	Allele range
EMVi029[a]	HEX-ACGAGTCCCAGGTCTCACAG	TGTTGACGGTCACGGTGTAT	164-248
Vica9/X[b]	FAM-TCGCGCATCACTATCTACAC	AGACAGGAATGTGGTGGAAG	219-247
Vica10/154[b]	HEX-CCTCCTTCCTATTACTCTCG	CTGAAGCGAACCTATGTCC	102-190
Vicacg8/42[b]	FAM-TGTCAGCCACGCTAGAAG	CACCGGACGAATCATGC	194-242
Vict1/130[b]	FAM-GATTGGTGACGCATGTGT	GCTGGAGATTGCGTAGAC	142-164
Vitc1/82[b]	HEX-ACTGTCTCTAGGCGAAAG	ACTTGGAAGCTCGCTAAG	225-243
Vitc2/16[b]	FAM-ACATTGACGAAGACGAGC	TACAATTGAGGCGTGTCC	153-169
Vitg9/129[b]	FAM-CTAATTCAACTCGCTGCGTC	TTTCAGCCAGCTAACCTAGG	277-293

[A] XU *ET AL*. (2009)
[B] GUERIN *ET AL*. (2004)

Determination of parentage

The parentage and family pedigree for each cultivar was assessed based on the National Fruit Collection database (DEFRA, 2010). It was not possible to assess Rosette as it was not in the

database. The database also did not include new cider cultivars Angela, Gilly, Lizzy, Three Counties, Tina and Vicky, which derived from a cross between Dabinett or Michelin with James Grieve or Worcester Pearmain (Liz Copas – personal communication). To confirm their parentage, we fingerprinted these new cultivars using the same SSR markers and methods as used for fingerprinting the National Fruit Collection (DEFRA, 2010) and comparing the allele sizes to those of the four possible parents from the collection.

Statistical analysis

Allele frequencies for an orchard were calculated using Powermarker software (Liu and Muse, 2005). Rare alleles ≤ 0.01 of the population of an orchard were recorded as missing values. If there were two alleles at a locus it was assumed that the lesion had resulted from infection by two different strains - only one was randomly selected for inclusion in analysis. If a sample had multiple loci with more than one allele then the sample was discarded. Null alleles occur when a mutation in the flanking region of the sequence repeat stops the annealing of the primer and therefore no amplification during PCR. In this study we included null alleles as a single allele for that marker although it is possible that the sequences in these samples differed.

To assess if scab populations on different cultivars within an orchard differ from each other, pairwise F_{ST} values were calculated and significance testing based on 1023 permutations using Arlequin version 3.5 (Excoffier and Lischer, 2010). Data from all sites, along with 2012 sample data from Ash Farm, Worcestershire, UK (a mixed orchard of Bramley, Cox and Worcester) (Passey *et al*. 2016), were then combined to calculate F_{ST} values, which were subjected to cluster analysis based on the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm using the software Mega (Tamura *et al.*, 2013).

RESULTS

Determination of Parentage

There is generally little known shared parentage among the cultivars sampled (potted and orchard trees) in the WM132 orchard (results not shown). Fiesta and Gala both have Cox in their genealogy; however most of the other cultivars are older, resulting from chance/raised seedlings. Of the cider cultivars in the Southwest of England Dabinett is from a chance seedling while Katy is from a Swedish cross between James Grieve and Worcester. Fingerprinting of "The Girls" showed that all six susceptible cultivars shared James Grieve as a common parent; Angela and Lizzy were from a cross between James Grieve and Michelin and the other four (Gilly, Three Counties, Tina and Vicky) from a cross of James Grieve with Dabinett.

General results

The numbers of isolates sampled varied greatly between cultivar and between sites due to differing scab severity (Tables 2 and 3). The difference between the number of isolates used in statistical analysis and the number of isolates with DNA extracted is due to the fact that a few samples failed to amplify or that a few samples had multiple alleles at more than one locus. Only one lesion was found on Dabinett in the Staunton-on-Wye orchard and therefore omitted from statistical analysis. There was no scab on the potted trees of Rosette in WM132 in 2013, or on the Dabinett trees placed in the same orchard in 2014.

Table 2 Number of apple scab isolates successfully screened with SSR markers from trees within WM132 orchard, Kent

Apple type	Cultivar sampled	2012[a]	2013[b]				2014[b]			
			Cox	Fiesta	Gala	Total	Cox	Fiesta	Gala	Total
Dessert	Cox	31(36)	25(27)	---	---	25(27)	---	---	---	---
	Fiesta	36(36)	---	35(35)	---	35(35)	---	---	---	---
	Gala	28(36)	---	---	35(36)	35(36)	---	---	---	---
	Golden Delicious	---	10(12)	9	14	35(37)	10	16	10	36
	Red Falstaff	---	14	9	14	37	8(9)	14(15)	6	28(30)
	Rosette	---	0	0	0	0	1	9(10)	2	12(13)
	Spartan	---	13	5	17	35	10	11(13)	10	31(33)
Culinary	Bramley	---	14	8	14	36	4(5)	6(10)	9(10)	19(25)
Cider	Angela	---	---	---	---	---	3	5	7	15
	Dabinett	---	---	---	---	---	0	0	0	0
	Somerset Redstreak	---	---	---	---	---	8	8	7	23

Number in the brackets is the number of isolates from which DNA was extracted; otherwise, all isolates with DNA extracted were used in statistical analysis. [a]2012 samples collected from orchard trees; [b]2013 and 2014 sampling from potted trees placed within the blocks of Cox, Fiesta or Gala orchard trees

Table 3 Number of apple scab isolates genotyped with SSR markers from commercial cider orchards in Southwest England

Staunton-on-Wye		Sandford	
Angela	10	Gilly	8
Dabinett	0(1)	Katy	6
Katy	29(36)	Three Counties	10
Lizzy	7(14)	Tina	26
Tina	20(35)	Vicky	26

Number in the brackets is the number of isolates from which DNA was extracted; otherwise, all isolates with DNA extracted were used in statistical analysis

Population pairwise comparisons

Pairwise F_{ST} values between populations from Fiesta and Gala in WM132 showed that the two populations differed significantly ($p = 0.03$) in 2012; however, this was not the case for the lesions from potted trees in 2013 ($p = 0.8$). The population from Cox was not different to that from either Gala or Fiesta in both years ($p > 0.05$).

Pairwise comparisons of the populations from Golden Delicious, Red Falstaff and Spartan showed they were significantly different in both 2013 and 2014 ($p < 0.05$; Table 4). The scab population from Golden Delicious did not differ from populations from the other cultivars. The scab population from Red Falstaff was different to that of Somerset Redstreak in 2014 ($p = 0.04$) and Bramley in 2013 ($p < 0.001$) and 2014 ($p = 0.01$). Although the scab population from Bramley in 2013 differed from that from either Golden Delicious or Spartan, there were no significant differences between these populations in 2014; nor between the Bramley population and any other cultivar's population (other than the aforementioned Red Falstaff). The only other cultivar that Spartan's scab population was different to was Angela's ($p < 0.001$). The population from Angela was not significantly different to that of any other cultivar, nor was that of the other cider cultivar Somerset Redstreak (other than that previously stated with Red Falstaff). The Rosette scab population showed no significant difference to any of the other cultivars.

Pairwise F_{ST} values between populations in the cider orchards are shown in Table 5. The only common cultivar comparison in the two commercial orchards in Southwest England was between the populations of Katy and Tina, which showed no significant difference in either orchard ($p > 0.1$). In the Staunton-on-Wye orchard, the population on Angela was significantly different to that of Lizzy ($p = 0.04$) and Tina ($p = 0.01$) and close to significance with the population from Katy ($p = 0.06$). There were no other significant pairwise differences. The scab population on Three Counties differed ($p < 0.001$) from the other four cultivars sampled in the Sandford orchard. The population on Gilly was close to being significantly different to that of Vicky ($p = 0.07$) and differed from that of Tina ($p = 0.01$).

A UPGMA tree (Figure 1) illustrates the differences between all populations in this study, including those 2012 samples from another mixed orchard as reported in Passey *et al*. (2016). The result indicates a general geographical difference between scab populations in the sampled orchards and a greater difference between populations on cider cultivars than on desert cultivars.

Table 4 Estimated pairwise F_{ST} values from AMOVA between scab isolates sampled from potted trees of different cultivars placed within the WM132 mixed cultivar orchard: upper diagonal - 2013 samples; lower diagonal - 2014 samples

	Angela	Bramley	Golden Delicious	Red Falstaff	Rosette	Somerset Redstreak	Spartan
Angela		---	---	---	---	---	---
Bramley	0.00		0.08***	0.07***	---	---	0.03*
Golden Delicious	0.01	0.00		0.07***	---	---	0.04**
Red Falstaff	0.01	0.04*	0.03**		---	---	0.03*
Rosette	0.00	0.00	0.00	0.02		---	---
Somerset Redstreak	0.01	0.00	0.01	0.03*	0.00		---
Spartan	0.07***	0.01	0.03**	0.05***	0.00	0.02	

* Significant at 0.05; ** Significant at 0.01; *** Significant at 0.001

Table 5 Estimated pairwise F_{ST} values from AMOVA of scab isolates sampled from different cultivars in mixed cider cultivar orchards in Southwest England: upper diagonal Staunton-on-Wye - 2012 samples; lower diagonal Sandford - 2014 samples

	Angela	Gilly	Katy	Lizzy	Three Counties	Tina	Vicky
Angela		---	0.05	0.13*	---	0.11*	---
Gilly	---		---	---	---	---	---
Katy	---	0.00		0.03	---	0.02	---
Lizzy	---	---			---	0.04	---
Three Counties	---	0.47***	0.48***	---		---	---
Tina	---	0.09*	0.03	---	0.39***		---
Vicky	---	0.04	0.00	---	0.36***	0.00	

* Significant at 0.05; ** Significant at 0.01; *** Significant at 0.001

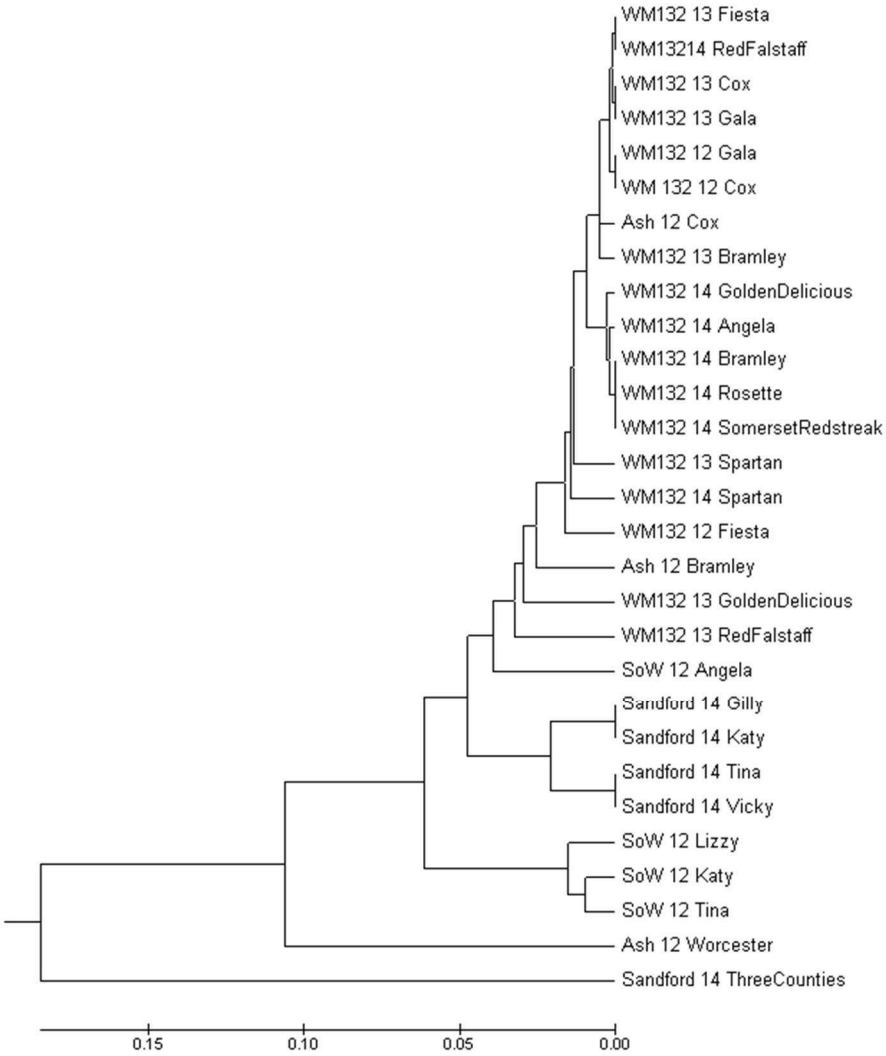

Figure 1 A UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tree generated from pairwise F_{ST} values of scab isolates sampled from different cultivars in three mixed orchards in three years; Population nomenclature: Orchard (SoW = Staunton-on-Wye), Sampling year, Cultivar

DISCUSSION

In this study we have shown that there are significant differences between some scab populations from different cultivars in the same orchard. However, it should be noted that population differences between orchards were much greater than between cultivars in the same orchard.

A number of populations from different cultivars were not different, suggesting that these cultivars probably share similar genetic factors of resistance (and therefore susceptibility) to

V. inaequalis and would therefore not make desirable candidates for use in a mixture for scab management. Thus, in desert apple it would not be advisable to plant Cox, Fiesta or Gala in the same orchard if the aim is to reduce scab development. On the other hand, Golden Delicious, Red Falstaff or Spartan are good candidates for such a purpose.

For the cider cultivars there were no significant differences in the scab populations from Katy, Tina and Vicky, but there was a large difference in the population from Three Counties with the populations of all other cider cultivars. This is surprising given that Gilly, Tina, Vicky and Three Counties all come from the same cross (Dabinett x James Grieve). In fact, of the 24 "The Girls" cultivars in the Sandford orchard we found a sufficient amount of scab for sampling on only four. All "The Girls" cultivars are from four possible crosses indicating that there is likely to be considerable differences in genetic control of the response to scab within the parents, leading to segregation of this character in the progeny. There were no scab lesions on potted trees of Dabinett in the WM132 orchard and only one lesion in the Staunton-on-Wye orchard; however, four of the six "The Girls" cultivars sampled had Dabinett as a parent. Worcester is likely to have at least three resistance factors (Barbara *et al.*, 2008) and the population on Worcester is quite different to the other populations presented. These results indicate that selection of cultivars for inclusion in mixture cannot be reliably made based solely on pedigree information.

The greater differences between scab populations on cider cultivars compared to the differences between desert cultivars suggest that there is less variability in resistance factors among the desert cultivars in the present study. Breeding for improved dessert cultivars has been much more extensive than cider apples, which may have reduced genetic variability among commercial desert cultivars for scab resistance. It would be advantageous to expand the genetic base from which desert cultivars are bred to increase the pool of resistance factors to *V. inaequalis* and potentially other pathogens.

The use of SSR markers is a good way to initially assess how similar populations of scab are from different cultivars and therefore discount those susceptible cultivars which show no differences as being incompatible for a successful mixed orchard for disease management. Of course, at this stage those cultivars that showed limited scab development remain viable candidates for mixture. It is possible to select potential mixture components based only on the SSR results when scab populations from those cultivars are different. Nevertheless, some controlled cross inoculation with isolates from these candidates may be advisable. Of course, any susceptible cultivar can be mixed with resistant cultivars.

The selection of cultivars for mixture is not decided on scab resistance alone, other traits have to be considered in order to maximise profitability. For example, Red Falstaff flowers almost two weeks before Golden Delicious and Spartan, which may impose extra cost in orchard management, e.g. pest and disease control. There is also some evidence that mixed cultivar apple orchards might reduce levels of powdery mildew and rosy apple aphid (Parisi *et al.*, 2013) and therefore comparing and selecting cultivars with differing resistance to other pests and diseases will further increase the benefit of mixture.

The cider industry remains the most obvious area of apple production for implementation of mixed cultivar orchards. Not only is fruit quality not as stringent as for desert apples, much mass produced cider includes a blend of apple cultivars and therefore requires different cultivars to be grown, as opposed to desert growers who are often more restricted in which cultivars they grow for the mass market. As we have shown in this study cider growers also seem to have the advantage of a more diverse genetic background in cultivars for reduction in scab.

ACKNOWLEDGEMENTS

This research was funded by the Department for Environment, Food and Rural Affairs (DEFRA) and the National Association of Cider Makers (NACM). We would like to thank Joyce Robinson for help in sampling and Robert Saville for comments. Thanks are extended to Liz Copas for information on "The Girls" collection. Thanks also to Thatchers Cider and Bulmers (Heineken) for access to their orchards for sampling of scab.

REFERENCES

BARBARA DJ. ROBERTS AL. XU X-M. (2008). Virulence characteristics of apple scab (*Venturia inaequalis*) isolates from monoculture and mixed orchards. *Plant Pathology,* **57**:552–561.

BERESFORD RM. MANKTELOW DWL. (1994). Economics of reducing fungicide use by weather based-disease forecasts for control of *Venturia inaequalis* in apples. *New Zealand Journal of Crop and Horticultural Science,* **22**:113–120.

BERRIE A. XU X-M. (2003). Managing apple scab (*Venturia inaequalis*) and powdery mildew (*Podosphaera leucotricha*) using Adem™. *International Journal of Pest Management,* **49**:243–249.

BLAISE P. GESSLER C. (1994). Cultivar mixtures in apple orchards as a mean to control apple scab? *Norwegian Journal of Agricultural Sciences,* **17**:105–112.

DEFRA. (2010). *Fingerprinting the National Apple and Pear Collections - GC0140*. Department for Environment, Food and Rural Affairs (Defra).

DIDELOT F. BRUN L. PARISI L. (2007). Effects of cultivar mixtures on scab control in apple orchards. *Plant Pathology,* **56**:1014–1022.

EXCOFFIER L. LISCHER HEL. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources,* **10**:564–567.

GUÉRIN F. FRANCK P. LOISEAU A. DEVAUX M. LE CAM B. (2004). Isolation of 21 new polymorphic microsatellite loci in the phytopathogenic fungus *Venturia inaequalis*. *Molecular Ecology Notes,* **4**:268–270.

KOCH T. KELLERHALS M. GESSLER C. (2000). Virulence pattern of *Venturia inaequalis* field isolates and corresponding differential resistance in *Malus* x *domestica*. *Journal of Phytopathology,* **148**:357–364.

LIU K, MUSE S V, 2005. PowerMarker: an integrated analysis environment for genetic marker analysis. *Bioinformatics,* **21**:2128–2129.

MUNDT CC. (2002). Use of multiline cultivars and cultivar mixtures for disease management. *Annual Review of Phytopathology,* **40**:381–410.

PARISI L. GROS C. COMBE F. PARVEAUD C-E. GOMEZ C. BRUN L. (2013). Impact of a cultivar mixture on scab, powdery mildew and rosy aphid in an organic apple orchard. *Crop Protection,* **43:**207–212.

PARISI L. LESPINASSE Y. GUILLAUMES J. KRÜGER J. (1993). A New Race of *Venturia inaequalis* Virulent to Apples with Resistance due to the *Vf* Gene. *Phytopathology,* **83**:533–537.

PASSEY TAJ. SHAW MW. XU X-M. (2016). Differentiation in populations of the apple scab fungus *Venturia inaequalis* on cultivars in a mixed orchard remain over time. *Plant Pathology,* Doi: 10.1111/ppa.12492.

ROBERTS AL. CRUTE IR. (1994). Apple scab resistance from *Malus floribunda* 821 (*Vf*) is rendered ineffective by isolates of *Venturia inaequalis* from *Malus floribunda*. *Norwegian Journal of Agricultural Sciences,* **17**:403–406.

SIEROTZKI H. EGGENSCHWILER M. BOILLAT O. MCDERMOTT JM. GESSLER C. (1994). Detection of variation in virulence toward susceptible apple cultivars in natural populations of *Venturia inaequalis*. *Phytopathology,* **84**:1005–1009.

TAMURA K. STECHER G. PETERSON D. FILIPSKI A. KUMAR S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution,* **30**:2725–2729.

XU X. ROBERTS T. BARBARA D. HARVEY NG. GAO L. SARGENT DJ. (2009). A genetic linkage map of *Venturia inaequalis*, the causal agent of apple scab. *BMC Research Notes,* **2**:163.