Project title:	The epidemiology and control of heuchera rust
Project number:	HNS 191
Project leader:	Dr Erika Wedgwood
Report:	Final Report, February 2016
Previous report:	Annual Report, February 2015
Key staff:	Erika Wedgwood (ADAS) Rebekah Robinson (RHS) Beatrice Henricot (RHS) Sarah Mayne (ADAS) Steve Richardson (ADAS) Gerard Clover (RHS)
Location of project:	ADAS Boxworth, Boxworth, Cambs, CB23 4NN Royal Horticultural Society, Wisley, Surrey, GU23 6QB
Industry Representative:	Neil Alcock Seiont Nurseries, Gwynedd, LL55 2BB
Date project commenced:	1st March 2014
Date project completed (or expected completion date):	28 February 2016

DISCLAIMER

AHDB, operating through its AHDB division seeks to ensure that the information contained within this document is accurate at the time of printing. No warranty is given in respect thereof and, to the maximum extent permitted by law the Agriculture and Horticulture Development Board accepts no liability for loss, damage or injury howsoever caused (including that caused by negligence) or suffered directly or indirectly in relation to information and opinions contained in or omitted from this document.

The results and conclusions of the experiments in this report are based on some investigations 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.

Dr Erika F. Wedgwood

Research Scientist

ADAS UK Ltd

Signature  Date 26 February 2016

Dr Rebekah Robinson

Research Scientist

The Royal Horticultural Society

Signature  Date 26 February 2016

Report authorised by:

Dr Barry Mulholland

Head of Horticulture

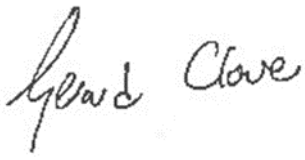
ADAS UK Ltd

Signature .  Date 26 February 2016

Dr Gerard Clover

Head of Plant Health

The Royal Horticultural Society

Signature  Date 26 February 2016

CONTENTS

Headline 7

Background 7

Summary 8

Financial Benefits 11

Action Points 12

General Introduction 13

Objective 1: To survey and review information relevant to heuchera rust incidence, and leaf sampling for rust species determination 14

Introduction 14

Methods 14

Results 15

Discussion 19

Objective 2: To determine if and where latent rust infection can be detected on heuchera plants during commercial production 20

Introduction 20

Objective 2.1: To refine and apply a molecular test to confirm if the current infection is caused by *Puccinia heucherae*, or another rust species. 21

Methods 21

Results 28

Discussion 38

Objective 2.2: To apply the molecular test and collect and grow on heuchera plants to determine whether plants arriving at nurseries are infected by *P. heucherae* ... 40

Introduction 40

Methods 40

Results 47

Discussion 50

Objective 3: To determine the mode of infection by *P. heucherae* and the environmental conditions required for the infection of heuchera plants 52

Introduction 52

Methods 53

Results 53

Discussion 53

General Conclusions 54

Knowledge and Technology Transfer 55

Glossary 56

References 56

Appendices 59

GROWER SUMMARY

Headline

Puccinia heucherae has been confirmed by molecular testing as the rust species present on UK nurseries.

Overwintered heuchera are an important source of inoculum, but reducing leaf wetness and preventative fungicide programmes can prevent rust infection

Background

Heuchera rust, caused by *Puccinia heucherae*, was first identified in the UK in gardens in 2004 and in nurseries in 2005. From 2010 to 2012, the incidence of the disease in nurseries and gardens (RHS advisory data, unpublished) increased significantly on a range of species and varieties. Information received from growers suggested that the rust has proven difficult to eliminate from nurseries. In the last three years many growers have used a combination of fungicides and cultural control methods, and so by 2015 symptoms were being seen less frequently where control measures were kept up. However, the limit to the number of fungicide applications of azoles (FRAC 11) and succinate dehydrogenase inhibitors (FRAC 7) for chemical resistance management means that covering the whole growing period from April to October is difficult, and likely to become harder with legislation changes, either not re-registering, or removing products from the market, in addition to the threat of loss through resistance. Products Folio Gold (chlorothalonil + metalaxyl-M (FRAC M5 + 4) and Fubol Gold (mancozeb + metalaxyl-M (FRAC 3 + 4) are available against rust at present, but resistance is known to metalaxyl-M by strains of Impatiens downy mildew, lettuce and onion downy mildews and potato blight. There have been a number of AHDB reports on fungicide control of rusts e.g. PC 057a, PC 185, PC 292, BOF 033 on chrysanthemum white rusts and HNS 106 on rose rust.

A principle query from UK heuchera growers is how and why crops are now becoming infected by rust. More information about the recent heuchera rust problem in the UK, e.g. material sources, disease incidence in relation to plant growing conditions, were needed, which could then be used by growers to prevent or reduce the establishment of rust on nurseries. Observations from growers and experiments together with information on other *Puccinia* species, was needed to gain greater understanding of the conditions favouring the pathogen and the potential source/s of the pathogen on nurseries. Symptoms are often reported in heuchera two to five months after potting and it is conjectured that this is from latent infection.

Work on chrysanthemum rust has shown detection by molecular testing four days post-infection, 10 days before any visible symptoms. By developing a molecular test for *P. heucherae* it should be possible to confirm if this is the species affecting UK heucherae and whether the rust is present in the supply chain as a symptomless infection.

Summary

The majority of heuchera growers from across England and Wales surveyed in 2014 had seen rust on plants at some time. No particular husbandry differences were found for the five out of 18 nursery sites which had not seen rust, compared with those where it had been found. Most growers used a number of plug suppliers (with 18 named). Most of the 73 varieties grown (UK growers have not seen rust on 48 of the varieties) were bred by Terra Nova in the USA. The varieties on which rust was most commonly reported by some growers were those grown more frequently such as Peach Flambé, Obsidian, Marmalade and Key Lime Pie, although others such as Plum Pudding, Green Spice, Palace Purple and Fire Chief were also frequently grown and not seen with rust. A Terra Nova breeder reported that the lighter, more recessive foliage colours and those with very thin leaves get the worst rust. Not much yellow foliage is bred now and selection is being made for tougher, thicker lime-coloured leaves.

All the UK growers consulted recognised that prolonged periods of leaf wetness and high humidity from close spacing and less-open growing media could encourage rust. The grower at Terra Nova in the USA lets heuchera flag before watering, to increase resistance to rust, possibly through increased cuticle thickness. Fungicides including azoxystrobin, propiconazole and myclobutanil were used routinely. Several UK growers had started to use preventative fungicide programmes including the same actives, but in one instance, once these stopped in October, severe rust was seen within a fortnight across many varieties. Latent rust may have become symptomatic or possibly the mild weather with morning dew in October may have favoured infection once fungicides ceased and allowed pustule formation.

Overwintered plants were consistently highlighted by growers as having a problem with rust, and are likely to be a major source of infection of new stock. Only one spore type, the teliospore, was seen to be produced in plants held under observation from 2014 to 2015. No *P. heucherae* infection developed in growth cabinets tests in 2014, and so the environmental requirements of *P. heucherae* for infection were unable to be determined. White rust of chrysanthemum, *Puccinia horiana*, also only has teliospores. Teliospores bud off basidiospores which can infect within five hours of dispersal at an optimum of 96% relative

humidity and 17-24°C, with symptoms within 10 days. Teliospores of white rust can survive for up to eight weeks on detached leaves.

In 2014, samples of rust-infected heuchera leaves were collected from ten different nurseries and garden centres and the rust was determined to be *P. heucherae* through DNA sequencing. A molecular PCR test was developed to detect the rust on symptomless plants.

Heuchera plants were sampled throughout 2015 across five supply chains at various growth stages: seedlings, micro-propagated plants, plugs/liners, retail plants and overwintered plants (Figure 1). This involved four micropropagators and one seed supplier who supplied four different nurseries. The supplied material was then collected from the nurseries as it grew into plugs and then 1 L container plants. Overwintered plants were originally micropropagated in 2014, but were from the same sources at each nursery as the 2015 produced plants. Plants were all visibly healthy on arrival and were tested for non-symptomatic rust infection using the molecular test. Plants were then grown in a controlled environment glasshouse and outdoors with overhead irrigation and inspected weekly for subsequent development of rust symptoms.

Rust was not detected in 2015 in either micro-propagated heuchera plants, plug plants from four supply chains (cv. Marmalade) or in seed-grown plants (cv. Palace Purple) from a fifth supplier. These plants at the start of the supply chain are unlikely to be the source of heuchera rust infections. Using the molecular test, rust was detected in four out of 36 symptomless overwintered heuchera (cv. Marmalade), collected from a nursery, and of these one developed a rust pustule on a leaf five weeks after quarantine in a glasshouse.

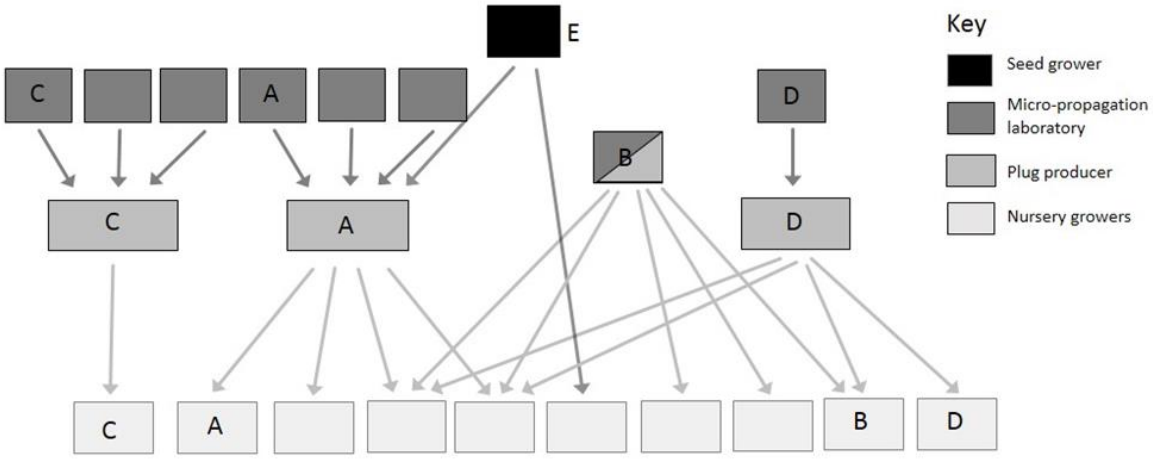


Figure 1. Schematic illustrating the complexity of the heuchera supply chain within the UK for a sample of 10 surveyed herbaceous nurseries. Nurseries A to E supplied material for testing for *P. heucherae* from each of the supply levels/plant stages shown in the key.

The likely lifecycle of *P. heucherae,* based on information about other species of *Puccinia* is shown in Figure 2 below. Conditions for basidiospore release from the brown teliospores held tightly in pustules on heuchera, are likely to be best when rain or irrigation splash can aid dispersal and when surfaces can remain wet for about five hours to aid spore survival and infection. As infection is favoured between 17 to 24°C, spring and autumn in the UK are likely to be the most favourable periods for infection. From molecular tests carried out in this project it is likely that the pathogen does not move through the plant from the infection point and so picking off individual leaves with pustules from plants when rust is first seen will prevent rust spread to other leaves on the same plant. Symptoms are likely after fungicide programmes finish in the autumn. Plants may take a month to develop symptoms after infection.

Infection is most likely to arise from overwintered plants, particularly as the dense canopies of finals will provide good infection conditions. Careful inspection of plants is required as dark spots visible from above caused by *P. heucherae* on older leaves can be attributed to other causes, and on the underside pustules can become flat. Some pustules drop out to leave only dark-rimmed holes. Trimming will remove infected leaves and reduce the chance of spores being produced in spring that will infect healthy tissue on the same and other plants.

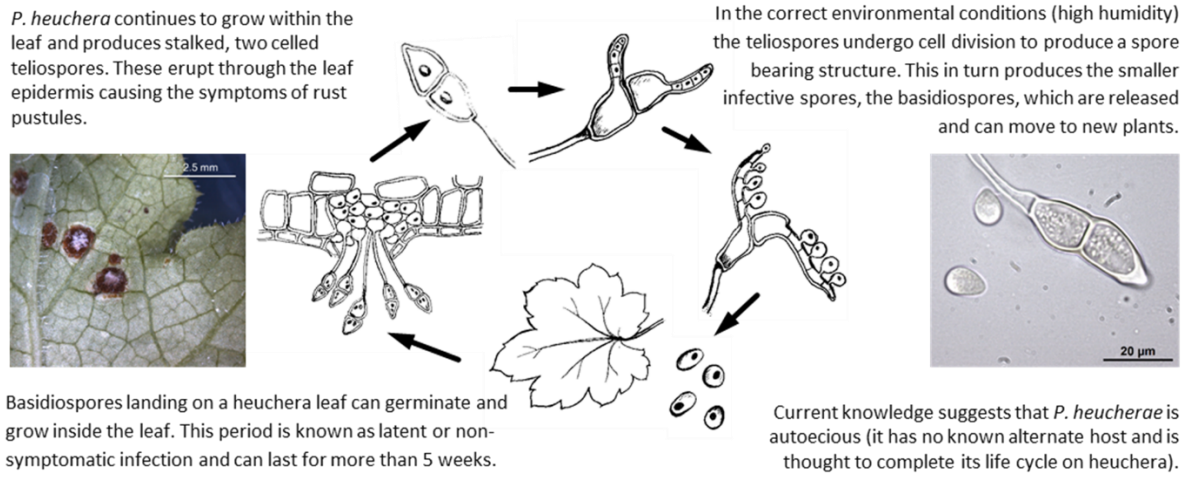


Figure 2. Probable lifecycle of *Puccinia heucherae*, with one host and two spore stages.

Financial Benefits

This project has provided growers with information on cultural control measures. These could mean fewer fungicide applications – currently some treat fortnightly over the six months from April. If all overwintered inoculum source is removed then in theory no sprays would be needed. For outdoor crops in drier years then timing watering so water does not stand on leaves should also mean no infection in these low risk situations. Allowing plants to flag to build up tissue resistance to infection may further reduce the chance of rust developing.

Information from growers found the variety Peach Flambé infected by rust by a greater proportion of producers and it may need to be avoided or grown using preventative fungicides. Growers with rust occurring on their nurseries could still continue to profit from heuchera, but could save plant losses by selecting varieties such as Palace Purple, Berrie Smoothie and Plum Pudding for their low chance of rust developing. However, many popular and so profitable lighter-coloured leaved varieties were rust-free on some nurseries and so do not need to be dropped by others from production. No plants from micropropagation or seed production contained *P. heucherae.* This should give propagators and growers confidence that micro-propagated and seed produced plants start off clean and so allow them to continue to source and sell popular varieties. A second benefit of the molecular test is that it has shown that if visibly infected leaves can be removed from a plant then there is unlikely to have been systemic spread within it. The plant could be sold without any justification for a refund request if rust is subsequently seen. Awareness by growers that the rust can be symptomless in the tissue for at least a month after infection means that growers prepared to quarantine can either

seek redress from their suppliers if rust develops and/or apply fungicide before pustules develop further and reduce marketability.

Action Points

- Reject any incoming plants showing symptoms of rust infection
- Aim to quarantine plants (or a sample of each variety) for a month after arrival, without fungicide application, in order to allow symptoms to show
- Regularly (at least weekly) inspect the foliage of all heuchera batches on the nursery closely (in particular leaf undersides) for rust pustule formation
- Remove old, senescing leaves from plants in autumn to help to prevent the fungus from overwintering and infecting new growth in spring. Thining will also facilitate leaf drying and remove surface moisture favourable to infection.
- Avoid watering overhead, or if this is not possible then water in the morning so that the leaf surfaces dry out rapidly, so that rust spores are unable to infect
- Improve air circulation around plants to speed leaf surface drying
- Let plants go very dry before irrigating. Irrigation should resume when first symptoms of wilting or ‘flagging’ appear
- If rust is seen then, if able to do so when under protection then raise temperatures to above 21° C during the day, and 15° C at night to produce conditions less favourable to the rust
- Use a preventative fungicide programme, especially if there has been overhead irrigation, and/or periods of rain on outdoor plants
- Avoid growing varieties that continue to be seen with infection, as they may have greater susceptibility to rust than other varieties. Darker varieties such as Palace Purple, Berrie Smoothie and Plum Pudding are popular and no rust has been seen, in contrast to the paler variety Peach Flambé, for example.

Molecular testing for latent infection of *P. heucherae* is now possible and could be used in propagation, and growers should let AHDB know if this would be useful so that work can be done to develop a service.

SCIENCE SECTION

General Introduction

Heuchera rust (*Puccinia heucherae*) was first recorded in UK gardens in 2004 and in nurseries in 2005 (Henricot *et al.,* 2007). *P. heucherae* is widespread in the USA and East Asia (Farr *et al.,* 2006 and Savile, 1954) but there were no previous records of rust disease recorded on *Heuchera* in the UK. In the UK the closely related rust, *Puccinia saxifragae*, has been found on the genus *Saxifraga* (Henderson, 2000) and in the USA this species has been recorded on *Heuchera* (Farr *et al.,* 1995).

The current research project resulted from increasing concern amongst some growers about the incidence and severity of rust on Heuchera over the last few years. A presentation on rusts on other crops and what was known about Heuchera rust was given at the July 2012 AHDB Herbaceous Perennials Technical Discussion Group summer meeting (Scrace, 2012). This highlighted five areas requiring further information; 1) the source / method of spread of Heuchera rust through the industry; 2) detailed epidemiology; including on the latent period; 3) survival in plant / on debris; 4) effective fungicides and good programmes; 5) resistant varieties (and potentially resistant strains).

The current project aimed to find out more about heuchera rust by further searching of publications and web-sites and by contacting breeders, propagators, growers and retailers either by questionnaire, telephone or site visits. In addition, experiments were carried out to investigate the conditions required for heuchera rust infection, utilising knowledge of the epidemiology of a rust which had the same spore type: chrysanthemum white rust (*Puccinia horiana*). Information on leaf wetness periods could be used in cultural control measures.

Growers do not currently know whether non-symptomatic plants are uninfected or whether the pathogen is present but not expressing symptoms in the plant. The purpose of the molecular test would be to enable detection of *P. heucherae* on heuchera plants before symptoms are present. This should inform growers and demonstrate whether *P. heucherae* exists as a latent and systemic pathogen. After further development, it might be possible for growers producing heuchera to check on the health status of their material using the test.

The project aims were to investigate and improve the understanding of heuchera rust and its epidemiology, specifically:

- To determine the origin of the infection by heuchera rust
- To elucidate the conditions when heuchera rust is most likely to occur

Objective 1: To survey and review information relevant to heuchera rust incidence, and leaf sampling for rust species determination

Introduction

Work under this objective was principally carried out in 2014 and is given in detail in the Annual Report. Information summarised here provides a background to the work using diagnostics for tracking the appearance of rust in the production chain carried out in Objective 2.

Methods

Survey and sampling

Following on from work carried out by Brough (2013), which included information from a heuchera rust survey given to attendees of the February 2012 Herbaceous Technical Group, in March 2014 a questionnaire was prepared and distributed to growers registered with the AHDB as having herbaceous crops. Information on the sources of material and the varieties grown together with husbandry practices that might have a bearing on the incidence of rust infection reported including watering regimes, cutting back of foliage, overwintering and fungicide application were recorded. The data from the 18 respondents was then examined to determine if there were any correlations between the reports of rust on heuchera, the material grown and the crop management practices. In addition, the principle breeder of heuchera in the USA was also contacted for information regarding rust.

During 2014, using information from the questionnaire, nurseries were visited across the UK to examine the bed locations and growing conditions of heuchera to see if further clues could be gathered as to why some nurseries had greater rust problems than others. Samples of infected plants were removed and maintained outdoors without fungicides at ADAS Boxworth and examined at intervals to record the progress of the disease. Leaf samples from these plants and from further nurseries were examined at ADAS Boxworth to record the spore stage, and duplicate samples sent to RHS Wisely for PCR analysis to determine the species of rust present. At the end of 2015 leaf and crown material from some of the infested plants was also sent for PCR analysis to determine whether the rust infection had become systemic.

Literature review

Information on heuchera rust was sought from publications and websites and plant breeders Conor Carey and Janet Egger at Terra Nova were contacted to find out more information on heuchera rust incidence and control in the USA.

Results

Sources of information from nurseries and breeders

The information from the 2012 AHDB survey (Brough, 2013), discussions with growers and the results of the current 2014 survey were amalgamated and reported fully in the Annual Report, and are summarised below. Information on the varieties with and without rust and their husbandry from the 18 sites were summarised in three detailed tables in the Appendix together with further information presented as seven case studies. Observations of heuchera rust pustules on plants of the 14 varieties held at ADAS Boxworth were also provided. Details of the heuchera species grown and an extensive list of varieties and their breeders were also given in the Appendix.

Heuchera production

Heuchera is native to the USA where different species are found in a mixture of habitats. Many commercially available varieties are interspecific hybrids using multiple species as their genetic basis. There has been a very active breeding programme to develop new foliage colours and flower features. The hub of heuchera breeding is in the USA, but some commercially grown varieties also originate in France, the Netherlands, Belgium, and the UK. Most modern commercial varieties are under Plant Breeder's Rights (PBRs). Plug plant producers principally use varieties grown by micropropagation and reported sourcing from New Zealand, Costa Rica, Indonesia, Poland, The Netherlands, Ireland and Scotland. Many propagators grow a large number of different varieties and deliver to growers all over the UK. There has been good consumer enthusiasm for the plants in recent years and heuchera can comprise up to 5% of nursery stock in some instances.

Host Distribution, Symptoms and Epidemiology

Host distribution

In Europe, *Puccinia saxifragae* is a common disease on *Saxifragaceae* (the family to which heuchera species belong) and was reported in the UK on the genus *Saxifraga* by Wilson and Henderson (1966). In the USA, *P. saxifragae* has also been found on heuchera (Farr *et al*., 2013). Worldwide, *Puccinia heucherae* is a common rust on heuchera and has been recorded in the USA and East Asia (Farr *et al*., 2013), but until 2004 it had not been reported in the UK. It is difficult to differentiate between *P. saxifragae* and *P. heucherae* based on morphological characteristics, but the two species can now be differentiated by DNA analysis (Henricot *et al*., 2007) and the first UK rust report on heuchera has been confirmed to be caused by *P. heucherae*. Rust has now been reported on heuchera from gardens and nurseries across

England (as far north as North Yorkshire) and Wales. As part of the current project it has been determined that of the samples of rust infected heuchera leaves collected from English nurseries in 2014 and sent to the RHS for PCR testing, all 16 of the varieties (sourced from across eight nurseries) had *P. heucherae* infection. For details of this testing please refer to the Annual Report.

In the USA, cultural methods and fungicide control have been applied successfully to manage rust on heuchera (J. Berckerman, Dan Heims of Terra Nova, pers. comm.). Terra Nova are breeding for rust-resistant heucherella (e.g. heuchera x tiarella).

Symptoms

The rust symptoms observed in the current heuchera rust project photographed and described in detail in the Annual Report match those given for heuchera rust on the RHS website (https://www.rhs.org.uk/advice/profile?pid=774).

Epidemiology

Information on the biology of the pathogen is also given on the RHS website (https://www.rhs.org.uk/advice/profile?pid=774). The majority of other *Puccinia* species have complex lifecycles, involving up to five different spore types and often requiring two hosts. No record was found in the literature of either *Puccinia* species found on heuchera having any other spores besides teliospores and the basidiospores (which bud from the teliospores) nor an alternate host.

In general, for species of *Puccinia,* humid conditions cause teliospores to germinate and produce basidiospores, the spores which are carried by air currents and infect new host plants. Infection is favoured by wet or humid conditions and therefore in UK gardens, the disease is favoured by wet summers. Having wet foliage through the afternoon and evening is believed to invite heuchera rust to proliferate in greenhouses (Pavlich, 2013). There is no record of whether heuchera rust teliospores can infect the plant directly.

The fungus overwinters on any infected leaves remaining on plants. Old leaves are retained on plants for months, with pustules still visible on leaves that have been killed by the pathogen. New leaves emerging in spring soon produce rust pustules and it has been confirmed in the current project that heuchera rust has a latency period where the plant is infected but remains symptomless (for more details see Objective 2 in the current report). It is not known how long this period of latency can last, nor what triggers symptom expression, but in the current project a period of five weeks was confirmed before pustules developed on previously symptomless

leaves of overwintered plants. It is possible that bought in plants could be harbouring hidden infection. For information regarding epidemiology in the USA, see the annual report for this project from 2015.

Observations on-site of location of rust on nursery beds

Growers were asked for their observations on rust location on nurseries to try and identify the sources of infection, i.e. whether the infection resulted from infected plants brought onto the nursery or from wind or splash borne spore movement between older and newer batches of plants on the nursery. There was no obvious distribution pattern of plants with symptoms across beds, but pustules were common on finals being kept overwinter. The information from the site surveys were limited by the fact that where rust had been seen in the past, growers were now using preventative fungicide programmes to span the period between spring and winter, or in one case (for covered crops) in autumn/winter only.

It was hypothesized that there might be alternate hosts in the lifecycle of heuchera rust but no obvious candidate was identified based on observations from survey sites. Heuchera rust may resemble chrysanthemum white rust (*Puccinia horiana*) in having no alternate host and pustules that are only formed of teliospores. Fungicide programmes could be targeted to the season of spore production on any alternate host, but currently protection has to be ongoing, particularly as most crops receive overhead irrigation that provides the leaf wetness that is likely to be required for spore infection.

Susceptible varieties

The results of both the 2012 and 2014 grower questionnaires do not point towards any supply routes being any more affected than others and also that any heuchera variety can succumb to infection. Details from these are given in the Appendix tables of the Annual Report. Differences were reported by growers with gold and lime varieties considered the least tolerant. Pustules, or the necrosis on the upper leaf surface associated with the pustules underneath, were, however, more easily seen on paler, often thinner, leaved varieties such as Lime Rickey, Key Lime Pie and Lime Marmalade. Gold varieties with rust reported include Peach Flambé and Marmalade. Others such as Plum Pudding, Green Spice, Palace Purple and Fire Chief were also frequently grown and not seen with rust. True susceptibility of any particular variety was not confirmed, however, as gold and lime varieties were more frequently grown by nurseries and so the absence of rust on other varieties could be linked to the absence of the latter plants on the sites.

New varieties of heuchera are being bred in the USA and UK with the anticipation of incorporating rust resistance based on the use of material with a lower probability of showing rust symptoms. Janet Egger of Terra Nova in the USA has started breeding thicker-leaved lime coloured varieties to reduce rust susceptibility, and noted that, although yellow varieties become worst affected by rust, any heuchera variety can become infected to varying degrees.

Prevalence of rust on nurseries

Disease is usually not seen when the propagation material arrives at the nursery, but can be observed on 12 to 15-week old micropropagated plants, with growers reporting it to be most common around two months after potting. In addition, many growers overwinter plants and some then see infection in the spring. Rust is seen by growers at all stages of growth, from plug to final.

Most growers grow under protection but some grow outdoors all year round. It has been noted that incidence of rust is higher when plants are grown under tunnels. Growing under protection may produce softer growth more susceptible to foliar diseases.

End consumers of heuchera are likely to vary widely in their knowledge of gardening and plant husbandry, and these plants could represent a large reservoir of inoculum for the commercial trade. A limited number of plant protection products are available for home use, but include the rust actives Roseclear (myclobutanil) and Penzole 100 (penconazole).

Disease management: cultural practices

Detail on crop husbandry was requested in the 2014 survey, with the aim of identifying particular practices which may either increase or decrease disease prevalence. Growers found overwintered crops to have a greater probability of showing rust, and many endeavour not to overwinter plants if possible. Some now do not grow varieties they have had rust problems with previously. Even growers who utilise good cultural controls such as watering first thing in the morning, removing leaf debris and trimming plants before overwintering, have reported rust and so fungicide programmes appear to be necessary. Other control measures such as increasing tunnel ventilation, lowering humidity by increasing plant spacing, and using bottom watering are also likely to reduce the chance of rust development

Hygiene measures applicable to reduce infection by rust pathogens can be found in the AHDB Factsheet 23/00 (Scrace, 2000) based on project PC 175. Additional information on cultural controls for rust prevention can be found on the RHS website (https://www.rhs.org.uk/advice/profile?PID=774). For a comprehensive review of

recommendations for heuchera rust control, including measures taken by the heuchera rust breeder Terra Nova in the USA of allowing the plants to flag before watering, see the Annual Report for this project from 2015.

Disease management: Chemical control

In the UK, there are a range of treatments for rust control but none have been tested experimentally on heuchera rust. Terra Nova nurseries in the USA use azoxystrobin and propiconazole (amongst others) and these can also be used in the UK to spray against rust on protected and outdoor crops. For a more comprehensive review of chemical control of heuchera rust, refer to the Annual Report for this project from 2015, which includes a complete list of fungicides available for use on heuchera, including their FRAC numbers, approved crop situations, and maximum rates.

Chemical fungicides reported in the AHDB grower survey from 2012 (Brough, 2013) showed a wide range of products were being used, but the most frequent were strobilurins (Amistar and Signum) and triazoles (Systhane 20 EW, Bumper 250 EC and Octave). Good control was reportedly achieved with Amistar and Bumper EC, with Systhane 20 EW giving moderate control.

Discussion

It is not known how the first heuchera reported with rust in 2004 from an English garden came to be infected by *P. heucherae*. It is possible there was a private import of a plant with initially undetected rust. From the surveys, not all UK growers had seen rust on their heuchera by 2014 and it is possible that infection may be related to local garden infection sources. This project has found that heuchera rust is known in the USA on material grown by the major heuchera breeder (Terra Nova). Heuchera used to be produced commercially by splitting mature plants, but all the surveyed UK propagators of the current new varieties grow under Plant Breeders' Rights and obtain micropropagated plants (or seed for one variety) from licenced laboratories. There is a possibility that *P. heucherae* could be present as a symptomless (latent) infection and be capable of moving from the infection point into the new tissue (systemic) which is then used in micropropagation. That micropropagated plants might be the source of infection on nurseries was suggested by growers and this project sought to develop molecular diagnostics to test this hypothesis in the second year under Objective 2.

Objective 2: To determine if and where latent rust infection can be detected on heuchera plants during commercial production

Introduction

Although *P. heucherae* infection can result in rust pustules on the leaves or stems, it is thought that there is likely to be a period of non-symptomatic infection prior to the development of the rust pustule (known as latent infection). This period of non-symptomatic infection is known to occur in other rust species but has not been studied in *P. heucherae*. For example the soybean rust pathogen, *Phakopsora pachyrhizi*, can exist in this period of latent infection for as long as 60 days (Ward *et al*., 2012). In another example the rust fungus *Puccinia thlaspeos* has been shown to remain latent for up to 9 months (Kropp *et al.*, 1996). However, the incubation period for *Puccinia horiana* in susceptible chrysanthemum plants is 7-10 days, with teliospores being formed a few days later (Firman and Martin, 2008). This period of non-symptomatic infection means that heuchera plants may be arriving on a site with infection but the infection cannot be detected through visual examination of the plants. This uncertainty means that growers cannot be sure when infected heuchera plants are entering the supply chain.

One method to detect latent infection is to use a molecular test which detects the DNA of the rust fungus within the heuchera plant. The most common molecular tests are based on amplification of DNA (using a test called polymerase chain reaction or PCR).

When designing a molecular test it is important to determine the sensitivity of the test. If the test is not sensitive enough then it will not detect low levels of infection and infected plants will incorrectly be diagnosed as healthy plants. The sensitivity of the PCR was therefore determined during the development of the test.

It is also important to ensure that any molecular test will be specific to the heuchera rust pathogen, *P. heucherae*. If the test is not specific to *P. heucherae* then there is the possibility that false positive results may occur from other contaminating fungi in a sample. A PCR test was therefore designed with high specificity to *P. heucherae* DNA. The specificity of the PCR test to *P. heucherae* was determined using a computer simulation against online databases of fungal DNA sequences, and also through laboratory tests against DNA from non-target organisms.

The molecular test was then used to detect whether latent infection was occurring in heuchera plants and indicate whether infection is entering the supply chain undetected.

Objective 2.1: To refine and apply a molecular test to confirm if the current infection is caused by *Puccinia heucherae*, or another rust species.

Methods

Sampling of nurseries and examination of rust pustules from affected plants

Infected plants were sampled from eight counties and 10 nurseries across the UK during the surveys carried out in Objective 1 in 2014 (Table 1). DNA was extracted from a pustule on an infected leaf using a QIAGEN DNeasy plant mini kit. The Qiagen DNeasy plant kit uses a column based silica-membrane and spin procedures to isolate and purify DNA. The plant material is frozen in liquid nitrogen and ground to a fine powder using a pestle and mortar. The ground sample is mixed with a lysis solution which disrupts the cells releasing the DNA. An enzyme is added (RNase) which removes any contaminating RNA from the sample. The sample is then passed through a QIAshredder spin column which homogenises the lysed cells. The lysate is added to a buffer solution and loaded onto a spin column with a silica membrane. The DNA in the sample binds to the silica membrane in the column and wash solutions are used to remove any contaminants in the DNA. The pure bound DNA is then re-suspended in a low salt buffer and is ready for use.

Table 1. Heuchera plants with visible rust infection, sampled from across the UK in 2014

Sample ID	Heuchera variety	Sample location	Nursery code sample source
A4	Heuchera 'Beauty Colour'	Surrey	Q
A5	Heuchera 'Ruby Bells'	Hereford	R
A6	Heuchera 'Obsidian'	Cheshire	S
A7	Heuchera 'Hollywood'	Cheshire	T
A8	Heuchera 'Chocolate Ruffles'	Cambridgeshire	U
A9	Heuchera 'Lime Rickey'	Cambridgeshire	U
A10	Heuchera 'Plum Pudding'	West Sussex	V
A11	Heuchera ' Georgia Plum'	West Sussex	V
A12	Heuchera 'Obsidian'	Surrey	Q
A13	Heuchera 'Sugar Frosting'	Surrey	Q
A14	Heuchera 'Crème Brulee'	Norfolk	W
A15	Heuchera 'Paris'	Berkshire	X
A16	Heuchera 'Hollywood'	Nottinghamshire	Y
A17	Heuchera 'Hollywood'	Cheshire	Z
A18	Heuchera 'Sparkling Burgundy'	Cheshire	Z

Amplification of DNA for identification of the fungal pathogen

To identify the fungus causing this current rust infection, a molecular test with broad specificity was chosen and performed. The divergent regions D1 and D1 of the large ribosomal sub unit (LSU) gene were amplified using the broad range fungal primers LROR/LR6 and the amplification procedures described by Maier *et al*. (2003) (See Appendix 1 for a diagram of fungal ribosomal RNA gene structure). Ready-To-Go PCR beads (Promega, UK) were used for PCR amplification with a total volume of 25 µL and a final concentration of 0.4 pmol/µl of each primer. One microliter of extracted DNA was added as the template DNA.

The amplified products were run on a 1.5% agarose gel (Sigma, UK) containing 1 x SYBR Safe (Life Technologies, UK) as a DNA stain. Fragments of approximately the expected size (1000 bp) were gel-extracted using a QIAquick Purification Kit (Qiagen, Germany). The purified fragments were sequenced by Beckman Coulter Genomics (UK) using the primers LROR/L6. All DNA fragments were sequenced for both strands.

The identity for each DNA sequence was established by comparing percentage identity with sequences deposited in the GenBank databases (www.ncbi.nlm.nih.gov/blast) using BLASTN.

Objective 2.1.2: To develop a molecular test to detect the fungus *Puccinia heucherae* in symptomless heuchera plants

Amplification of the ITS region for P. heucherae with broad range primers

In order to design a molecular test with high specificity for *P. heucherae*, the variable DNA sequence in the Internal Transcribed Spacer (ITS) region of the fungal rRNA gene operon was sequenced.

ITS regions 1 and 2 were amplified using the primers ITS1-F and ITS4 which specifically amplify products from Basidiomycete fungi (Gardes and Bruns, 1993). Amplification, purification and sequencing were performed as for the amplification of the LSU gene as described previously.

Design of ITS PCR primers specific to P. heucherae

PCR primers were then designed to amplify the ITS region of *P. heucherae*, aiming to exclude all other possible contaminating organisms.

Literature searches provided identities of microbial contaminants which might be found on heuchera plants or within a nursery site (Table 2). ITS DNA sequences for these contaminants were retrieved from the NCBI DNA database and PCR primers were designed using a combination of DNA sequence alignments, the NCBI primer design function in GenBank (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and an Oligo Property Scanner (MWG Eurofins).

Six primers were designed to amplify DNA within the ITS region of *P. heucherae*. The specificity of each primer was tested against the fungal ITS DNA sequences available in the NCBI database, using the Primer BLAST function in GenBank.

Table 2. Identities of possible contaminants accounted for in the design of PCR primers for the molecular test to amplify DNA of *P. heucherae*

Botryosphaeria - cankers *Botrytis* – grey mould *Calonectria* – leaf blights *Cladosporium* – black moulds *Colletotrichum* – anthracnose fungi *Erysiphe* – powdery mildews *Fusarium* – wilt and rot fungi *Phyllosticta* – leaf spots *Podosphaera* – powdery mildews *Phytophthora* – blights, cankers & root rots *Plasmopora* – downy mildew	*Ramularia* – leaf spots *Udeniomyces* – yeast Rusts: *Gymnosporangium* *Kuehneola* *Melampsora* *Uromyces* *Puccinia*

Selection of PCR primers

Four primer pairs were selected for further analysis. These primer pairs were selected to provide a range of sizes of PCR product and to allow development of a nested or semi-nested PCR amplification if necessary. The binding positions of the selected primers within the Internal Transcribed Spacer (ITS) regions of the fungal rRNA gene operon are illustrated in the schematic in Appendix 1.

Design of a PCR primer pair to amplify heuchera plant DNA as a control reaction

A further primer pair was designed to amplify a 170bp product from the ITS region of heuchera plant DNA. This primer pair was included within reactions as a control to demonstrate that a negative result in a rust DNA PCR amplifications was not the result of poor quality or absent template DNA:

Heuchera-ITS-forward 5'- GGGACAAGGATTTTGTGGCG
Heuchera-ITS-reverse 5'- TGAGGATCATCCCGAGCAAC

A positive rust DNA control was also amplified with each batch of PCR reactions, alongside an amplification of a blank DNA extraction control and a no-template PCR negative control.

Optimisation of selected primer pairs

A gradient temperature block (56 ˚C – 68 ˚C) was used to determine the optimum PCR recombination, or annealing, temperature for each primer pair.

PCR reactions were performed using 1 x Bioline Biomix in a 25 µl reaction with 1 µl template DNA and 35 amplification cycles. A ten-fold dilution of a DNA extraction from 100 mg of *P. heucherae* infected heuchera leaf was used as the positive template DNA. The amplified products were run on a 1.5% agarose gel at 5V/cm and viewed under ultra-violet light

Testing primer sensitivity

Molecular tests require high sensitivity to detect low levels of infection within a plant. PCR primers bind to DNA with variable levels of efficiency. It is therefore important to determine the lower limit of detection for each primer pair in order to select the primer pair with the highest sensitivity. Two methods for assessing primer sensitivity were used: the first method used a serial dilution of pure *P. heucherae* DNA, and the second method used serial dilutions of infected and uninfected plant tissue. The first method provides a direct measurement of the detection limit of each primer pair for the target DNA, therefore providing a comparison of primer efficiency. The second method provides a more practical indication of the sensitivity of the primers to detect low levels of infection in heuchera plants. The percentage of infected tissue in non-infected tissue would equate to a mixture of infected and non-infected leaves in a sample to be tested. For example a detection limit of 0.01 % infected tissue would equate to an ability of the molecular test to detect 1 infected leaf mixed within a batch of 9999 non-infected leaves.

Primer pair sensitivity was initially assessed from the annealing (or DNA recombination) temperature optimisation PCR reactions. Primer pairs 2F and 4R, and 1F and 3R were discarded while primer pairs 1F and 4R, and 1F and 3R were selected for the higher resolution sensitivity testing. PCR sensitivity can be improved by performing two rounds of amplification in a nested or semi-nested PCR approach. A semi-nested PCR approach was therefore also tested with an initial amplification using the primers 1F and 4R and a second round of amplification using primers 1F and 3R.

Puccinia heucherae DNA extraction and serial dilution

Rust pustules were dissected under a low magnification and the teliospores were collected without contamination from plant leaf tissue. The rust teliospores were ground with a micro-pestle in DNA extraction buffer and the DNA was extracted using a QIAGEN DNeasy plant mini kit. The resulting rust DNA was quantified using a Qubit 3.0 and was diluted to a working concentration of 1 ng μl^{-1} DNA using sterile distilled water. A ten-fold serial dilution was made from the highest concentration of 1 x 10^{-9}g μl^{-1} DNA through to the lowest concentration on 1 x 10^{-18}g μl^{-1} DNA.

Serial dilutions of infected tissue and uninfected tissue

Leaves with rust pustules were collected and 1000 mg of leaf tissue was selected from areas in immediate proximity to a rust pustule but not containing teliospores. This material should contain mycelium of the rust fungus as would be expected in a latent asymptomatic infection. The leaf material was ground to a fine powder using a micro-pestle and liquid nitrogen. The powdered leaf material was suspended in 4 ml sterile distilled water. A suspension of 1000 mg leaf tissue in 4 ml sterile distilled water was also made using leaf material from an uninfected plant.

A ten-fold serial dilution of infected tissue in uninfected tissue was made from these leaf suspensions. Five dilutions were made; 10 % infected tissue, 1 % infected tissue, 0.1 % infected tissue, 0.01 % infected tissue, and 0.001 % infected tissue. DNA extractions were performed using 400 µl suspended tissue from each dilution (equivalent to 100 mg plant tissue per extraction). Extractions were made of the stock 100 % infected tissue and 100 % uninfected tissue samples. DNA was extracted using a QIAGEN DNeasy plant mini kit.

PCR reactions were performed for all dilutions of *P. heucherae* DNA and for all dilutions of infected material in uninfected leaf material.

Testing primer specificity in the laboratory

The theoretical specificity of the primers were determined through computer simulations using an online database of fungal DNA sequences. The specificity of the semi-nested PCR to *P. heucherae* was confirmed in the laboratory using template DNA from 18 possible contaminating organisms (7 rust, 11 non-rust) (Table 3).

Table 3. Fungi and oomycetes selected for testing specificity of the semi-nested PCR

Latin name of pathogen	Common name of pathogen
Rusts:	
Melampsora euphorbiae	Euphorbia rust
Puccinia buxi	Box rust
Puccinia heucherae	Heuchera rust
Puccinia lagenophorae	Groundsel rust
Puccinia pelargonii-zonalis	Geranium rust
Puccinia porri	Allium rust
Puccinia saxifragae	Saxifrage rust
Pucciniastrum sp.	Fuchsia rust
Other organisms:	
Armilaria gallica	Honey fungus
Botrytis cinerea	Grey mould
Colletotrichum sp.	Anthracnose
Erysiphe sp.	Powdery mildew
Golovinomyces biocellatus	Powdery mildew
Mortierella elongate	Endophytic fungus
Mycosphaerella sp.	Leaf spot
Phytophthora cinnamomi	Root rot
Pythium intermedium	Root rot
Ramularia sp.	Leaf spot
Podosphaera sp.	Powdery mildew

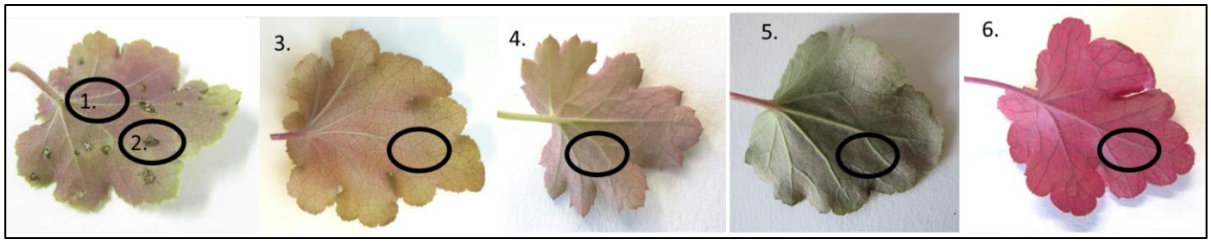
Preliminary tests for *P. heucherae* detection using the semi-nested PCR reaction

Amplification of P. heucherae DNA from a mixed DNA sample

Amplification of the large subunit (LSU) and internal transcribed spacer (ITS) DNA sequences from heuchera rust pustules using broad range primers, resulted in co-amplification of DNA from other contaminating fungi present on the heuchera leaf. This produced a number of poor quality sequence traces and amplification of *Udeniomyces* and *Colletotrichum* DNA from two samples (Table 5). This demonstrated the need for a molecular test which was specific *to P. heucherae*. The newly designed semi-nested PCR was tested on these mixed samples to determine if it could amplify a DNA product from *P. heucherae*, while excluding amplification of DNA from the other contaminating fungi.

Detecting P. heucherae infections in leaves without symptoms

The newly designed semi-nested PCR reaction was used in preliminary tests to determine its ability to detect infection in symptomatic (with pustules) and non-symptomatic (potentially with latent infection) heuchera plants. A selection of naturally, visibly, infected heuchera plants were selected for testing (Figure 3). Sample 2 was to test for latent infection that could either originate from growth of the pathogen within the leaf well beyond visible infection, or infection at the sample point that had not yet developed symptoms. In many diseases the pathogen moves out only a few millimetres in front of any visible damage in order to feed on fresh tissue. Where leaves were sampled without visible symptoms, but from a plant with pustules elsewhere (sample 3) this was to test whether the fungus was systemic within the plant i.e. the mycelium was inside tissue throughout the plant, but causing no visible symptoms.



1. Rust pustule 2. Adjacent to pustule cv. Marmalade	3. No pustule on mature leaf, but plant infected. cv. Marmalade	4. No pustule on young leaf, but plant infected cv. Marmalade	5. No pustule, no visible symptoms on plant cv. Green Spice	6. No pustule, healthy plant, 2 yrs quarantined cv. Marmalade

Figure 3. Examples of positions on leaves sampled to test the newly designed semi-nested PCR reaction and whether or not the heuchera plant had visible rust symptoms

Three leaf discs (1 cm diameter) were selected from each plant and washed vigorously in three rinses of sterile distilled water. The three leaf discs from each plant were then dried on sterile filter paper and ground to a fine powder using a micro-pestle and liquid nitrogen. A QIAGEN DNeasy plant mini kit was used to extract the DNA from each sample. PCR reactions were performed using 1 x Bioline Biomix in a 25 µl reaction with 1 µl template DNA and 35 amplification cycles. A semi-nested PCR reaction was used with primers 1F and 4R in the first amplification and primers 1F and 3R in the second amplification. The second round of amplification also included the newly designed primer pair ‘Heuchera ITS Forward’ and ‘Heuchera ITS Reverse’, to amplify a small 170 bp product from the ITS region of the heuchera plant. The amplified products were run on a 1.5% agarose gel at 5V/cm and viewed under ultra-violet light.

Confirming the specificity of the semi-nested PCR in the laboratory

A test was conducted to determine if the *P. heucherae* primers developed in this project cross-reacted with *Puccinia saxifragae.* Heuchera species belong to the Saxifrage family. In the UK an indigenous rust, *P. saxifragae*, occurs uncommonly on *Saxifraga* species. A search of the Kew fungal database and the USDA fungal database revealed that in the UK *P. saxifragae* has been recorded only on five *Saxifraga* species; *S. stelaris, S. spathularis, S. granulata, S. umbrosa* and *S. hyponoides,* and has not been recorded on other species in the family Saxifragaceae.

P. saxifragae samples were requested from the fungal herbarium at Kew. A small section of an infected sample was ground to powder with a micro-pestle and DNA extraction was performed using a QIAGEN DNeasy plant mini kit. The resulting DNA extraction was used as a template for semi-nested PCR amplification with the *P. heucherae* primers.

Confirmation of species of rust infecting *Heucherella* spp.

A planting of *Heuchera*, *Heucherella* and *Tiarella* species was examined in August 2015 in a garden in Surrey, UK. The herbaceous bed was planted in May 2013 with heuchera varieties Key Lime Pie (15 plants) and Mahogany (30 plants), heucherella cv. Stoplight (30 plants) and tiarella cv. Spring Symphony (30 plants). Key Lime Pie and Mahogany heuchera varieties both had large numbers of dark brown pustules which most likely indicate a heavy infection during the previous season. Similar dark brown rust pustules were also observed on heucherella cv. Stoplight.

Results

Objective 2.1.1: To refine and apply a molecular test to confirm if the current infection is due to *Puccinia heucherae*, or another rust species

DNA sequencing to identify the current heuchera rust pathogen

Morphological examination of an infected heuchera leaf with rust pustules and teliospores was carried out (Figure 4). Spore mounts were prepared in water and observed at x400 magnification with an optical microscope (Leica DM LB2).

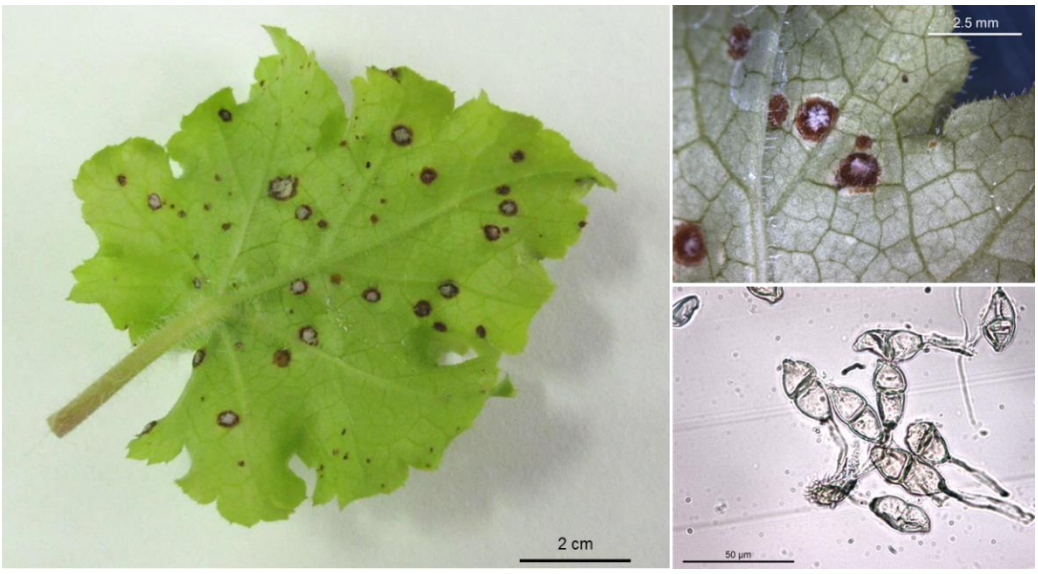


Figure 4. Heuchera rust pustules on leaves and high magnification of 2-celled teliospores

Analysis of the ribosomal large subunit DNA sequences extracted from 15 visibly affected plants confirmed the identity of the rust as *P. heucherae* (Table 4). Two of the samples which were tested produced poor quality sequence traces, indicating that the PCR primers were amplifying a product from contaminating fungal species in the sample.

Table 4. DNA analysis of the ribosomal large subunit. Identities are indicated as the closest sequence match in the NCBI database.

Sample ID	**Heuchera variety**	**LSU fragment**	
		Closest sequence identity	**% Match**
A4	Heuchera 'Beauty Colour'	*Puccinia heucherae*	100
A5	Heuchera 'Ruby Bells'	*Puccinia heucherae*	100
A6	Heuchera 'Obsidian'	*Puccinia heucherae*	100
A7	Heuchera 'Hollywood'	*Puccinia heucherae*	100
A8	Heuchera 'Chocolate Ruffles'	*Puccinia heucherae*	100
A9	Heuchera 'Lime Rickey'	*Puccinia heucherae*	100
A10	Heuchera 'Plum Pudding'	*Puccinia heucherae*	100
A11	Heuchera ' Georgia Plum'	*Puccinia heucherae*	100
A12	Heuchera 'Obsidian'	*Puccinia heucherae*	100
A13	Heuchera 'Sugar Frosting'	*Puccinia heucherae*	100
A14	Heuchera 'Crème Brulee'	*	*
A15	Heuchera 'Paris'	*	*
A16	Heuchera 'Hollywood'	*Puccinia heucherae*	100
A17	Heuchera 'Hollywood'	*Puccinia heucherae*	100
A18	Heuchera 'Sparkling Burgundy'	*Puccinia heucherae*	100

* Sequences had poor quality readings indicating the presence of multiple fungal species within the DNA extraction. Identities for these sequences were not confirmed.

Amplification of the ITS region for *P. heucherae*

The ITS sequence for *P. heucherae* has not previously been deposited in a public database. There is therefore no reference sequence for comparison in the NCBI database. The sequences that were confirmed as *P. heucherae* through the LSU sequence analysis most closely match the rust *P. veronicae-longifoliae* (93% identity; Table 5).

The non-specific nature of the primers used here also resulted in four poor quality trace sequences and two amplifications of products other than *Puccinia*. *Udeniomyces* are yeasts found on plant leaves, and *Colletotrichum* are fungal endophytes or pathogens often causing leaf spots.

Table 5. DNA analysis of the ITS regions. Identities are indicated as the closest sequence match in the NCBI database

Sample ID	Heuchera variety	ITS fragment	
		Closest sequence identity	**% Match**
A4	Heuchera 'Beauty Colour'	*Udeniomyces* sp.	99
A5	Heuchera 'Ruby Bells'	*	*
A6	Heuchera 'Obsidian'	*Puccinia veronicae-longifoliae*	93
A7	Heuchera 'Hollywood'	*Puccinia veronicae-longifoliae*	93
A8	Heuchera 'Chocolate Ruffles'	*Puccinia veronicae-longifoliae*	93
A9	Heuchera 'Lime Rickey'	*Puccinia veronicae-longifoliae*	93
A10	Heuchera 'Plum Pudding'	*Puccinia veronicae-longifoliae*	93
A11	Heuchera ' Georgia Plum'	*Puccinia veronicae-longifoliae*	93
A12	Heuchera 'Obsidian'	*	*
A13	Heuchera 'Sugar Frosting'	*Puccinia veronicae-longifoliae*	93
A14	Heuchera 'Crème Brulee'	*	*
A15	Heuchera 'Paris'	*	*
A16	Heuchera 'Hollywood'	*Colletotrichum acutatum*	100
A17	Heuchera 'Hollywood'	*Puccinia veronicae-longifoliae*	93
A18	Heuchera 'Sparkling Burgundy'	*Puccinia veronicae-longifoliae*	93

* Sequences had poor quality readings indicating the presence of multiple fungal species within the DNA extraction. Identities for these sequences were not confirmed.

Objective 2.1.2: To develop a molecular test to detect the fungus *Puccinia heucherae* in symptomless heuchera plants

Design of PCR primers specific to *P. heucherae*

PCR amplification of DNA requires a forward and a reverse PCR primer. Three forward primers and three reverse primers were designed to amplify DNA of *P. heucherae*, whilst excluding amplification of other possible contaminating DNA (Table 6).

The primers were grouped into pairs for PCR amplification and tested using a computer simulation for non-target binding against 33,559,332 fungal ITS sequences available in an online fungal database (NCBI, GenBank). Of the PCR primer pairs, three primer pairs were highly specific to *P. heucherae* and did not bind to any non-target sequences. The other two primer pairs had potential to amplify one non-target *Puccinia* species (Table 7). A further description of primer specificity is available in Appendix 2.

Table 6. Newly designed PCR primer sequences for amplification of *P. heucherae* DNA

Primer	Sequence (5' to 3')
1F	ATTGTGGCTCGACCCCTTTTA
2F	GCATCTTGCACCTTTTGGTATT
3F	GTGGATGTTGAGTGTTGCTGT
2R	ACCAAAAGGTGCAAGATGCG
3R	ACAGCAACACTCAACATCCAC
4R	AGTATTTGACTACTTTCCTTAATGC

Table 7. Newly designed PCR primer pairs and their theoretical amplification of DNA other than *P. heucherae* in a computer simulation

PCR Primer pair	Theoretical amplification of non-target DNA
1F and 2R	None
1F and 3R	None
1F and 4R	One non-target *Puccinia* species
2F and 3R	One non-target *Puccinia* species
2F and 4R	None

The primer pairs selected for further analysis are provided in Table 8.

Table 8. Selected primer pairs for further work and the corresponding size of the amplified DNA product

Primer pair	Predicted DNA product size (bp)
1F and 4R	530
2F and 4R	220
1F and 3R	430
1F and 2R	327

Optimisation of PCR reactions

A gradient temperature block (56 ˚C – 68 ˚C) was used to determine the optimum annealing temperature for use in the PCR reaction. The optimum annealing temperature for the PCR reaction was determined to be 62 ˚C for all primer pairs tested.

Determining the sensitivity of the molecular test

The lowest detection limits for each primer pair and for the semi-nested reaction were determined through gel image analysis (examples of gel images are shown in Figure 5) and are summarized in Tables 9 and 10.

Primer pair 1F and 4R was the least sensitive and had a detection limit of a 10 % infected material in uninfected leaf material. Primer pair 1F and 3R had higher sensitivity and with a lower detection limit of 0.01% infected material in uninfected leaf material. The semi-nested approach improved sensitivity 100 fold for detection of pure spores and 10 fold for the dilution series of infected leaf material and clean leaf material. These detection limits compare favourably with the 10^{-12} g detection limit for chrysanthemum white rust DNA using conventional PCR as determined by Alaei *et al*., (2009) and their 10^{-15} g detection limit for nested PCR.

Table 9. Sensitivity of the newly designed PCR primer pairs for amplification of *P. heucherae* DNA

PCR Primer Pair	Lowest level of *P. heucherae* DNA detected (g)
1F and 4R	10^{-13}
1F and 3R	10^{-13}
1F 4R and 1F 3R	10^{-15}

Table 10. Sensitivity of the newly designed PCR primer pairs for amplification of DNA extracted from dilutions of *P. heucherae* infected leaf material with uninfected leaf material to create decreasing amounts of the pathogen for detection

PCR Primer Pair	Lowest % of *P. heucherae* infected leaf detected by PCR
1F and 4R	10.00
1F and 3R	0.010
1F 4R and 1F 3R	0.001

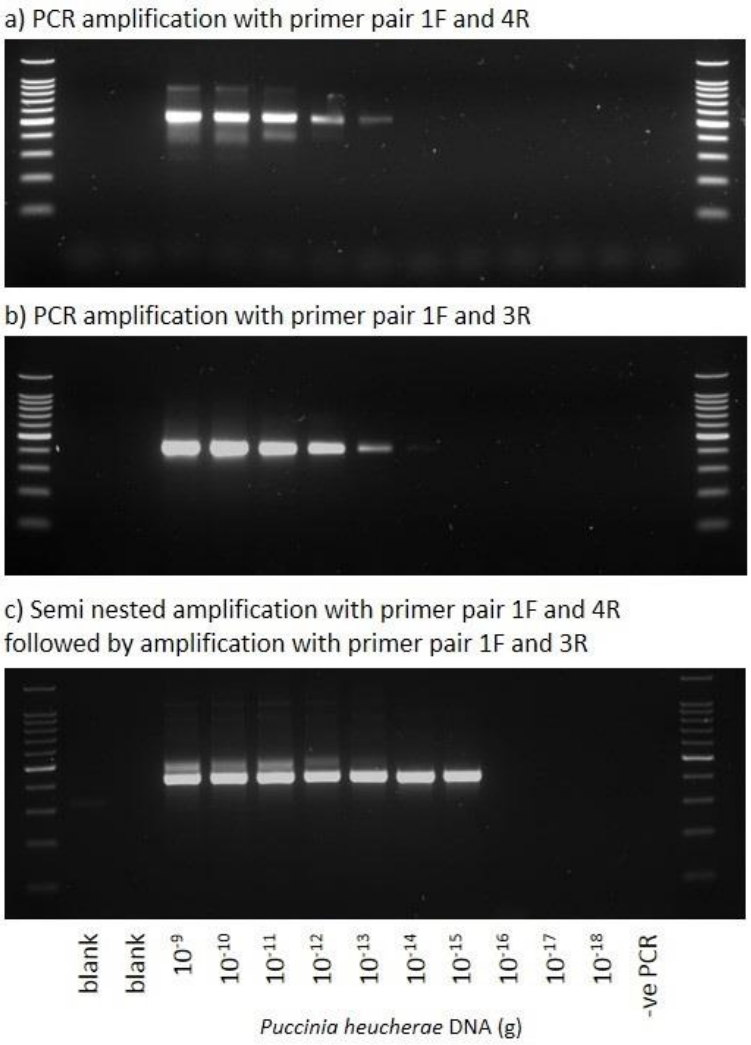


Figure 5. Example gel images for PCR amplification of *Puccinia heucherae* DNA dilutions using the newly designed primer pairs. The gel images indicate the reduction in the amplification of DNA as the concentration of the *P. heucherae* DNA is decreased. The semi-nested amplification (c) has the greatest sensitivity

Confirming the specificity of the semi-nested PCR in the laboratory

The specificity of the semi-nested PCR was tested in the laboratory using template DNA from 18 possible contaminating organisms (seven rust, 11 non-rust). No amplified PCR product was detected for any of the tested potentially contaminating organisms (Table 11), thereby supporting the results from the computer simulation during the design stage of the PCR primers. Only *P. heucherae* samples showed DNA amplification with semi-nested *P. heucherae* PCR primers.

To determine if the *P. heucherae* primers developed in this project cross-reacted with the indigenous rust common on *Saxifraga* species, *P. saxifragae,* a DNA extraction was made from the herbarium sample obtained from Kew. This was then used as a template for semi-nested PCR amplification with the *P. heucherae* primers.

No PCR product was detected from this amplification. However, the sample was also tested with the generic ITS primers, ITS1-F and ITS4 (Gardes and Bruns, 1993), and no PCR product was detected from this reaction (Table 11). This indicates that the DNA from a small section of the herbarium sample may not be suitable for PCR amplification. Further work and optimisation is therefore required before it can be determined whether the newly designed *P. heucherae* primers also amplify *P. saxifragae*.

Non-target DNA samples, other than for *P. saxifragae*, were shown to produce a PCR product when a non-specific ITS primer pair was used as a control in a separate reaction.

Table 11. Species tested against the newly designed semi-nested PCR for amplification of *Puccinia heucherae* DNA to confirm the test specificity.

Pathogen Latin Name	**Pathogen Common name**	**DNA amplification with semi-nested *P. heucherae* PCR primers**	**DNA amplification with generic fungal ITS PCR primers**
Rusts			
Melampsora euphorbiae	Euphorbia rust	No	Yes
Puccinia buxi	Box rust	No	Yes
Puccinia heucherae	Heuchera rust	Yes	Yes
Puccinia lagenophorae	Groundsel rust	No	Yes
Puccinia pelargonii-zonalis	Geranium rust	No	Yes
Puccinia porri	Allium rust	No	Yes
Puccinia saxifragae	Saxifrage rust	No	No*
Pucciniastrum sp.	Fuchsia rust	No	Yes
Other species			
Armilaria gallica	Honey fungus	No	Yes
Botrytis cinerea	Grey mould	No	Yes
Colletotrichum sp.	Anthracnose	No	Yes
Erysiphe sp.	Powdery mildew	No	Yes
Golovinomyces biocellatus	Powdery mildew	No	Yes
Mortierella elongate	Endophytic fungus	No	Yes

Mycosphaerella sp.	Leaf spot	No	Yes
Phytophthora cinnamomi	Root rot	No	Yes
Pythium intermedium	Root rot	No	Yes
Ramularia sp.	Leaf spot	No	Yes
Podosphaera sp.	Powdery mildew	No	Yes
* No detection possibly because the sample was not suitable			

Objective 2.1.2: Preliminary tests using the semi-nested PCR reaction in order to detect *P. heucherae* in symptomless plants.

Amplification of P. heucherae DNA from mixed samples

Previous amplification of large subunit (LSU) and internal transcribed spacer (ITS) DNA sequences from heuchera rust pustules with Basidiomycete-specific primers, resulted in co-amplification of DNA from other fungi present on the heuchera leaf. This produced a number of poor quality sequence traces and amplification of *Udeniomyces* and *Colletotrichum* from two samples (Table 5 in Objective 2.1). This demonstrated the need for a molecular test which was specific *to P. heucherae*. Amplification using the newly designed semi-nested *Puccinia*-specific ITS primers eliminated this problem and high quality sequences of *P. heucherae* were recovered for all samples.

Detecting P. heucherae infections in leaves without symptoms

The results from the preliminary PCR reactions demonstrated that rust could be detected in leaf tissue before symptoms of infection were visible (Table 12). This indicates that the molecular test can detect very low levels of infection in the plant. This is demonstrated by the positive amplification of *P. heucherae* DNA in a young leaf without pustules from an infected plant. This result may demonstrate the occurrence of localised symptomless infection points from spores landing on the leaf surface. It may also demonstrate that *P. heucherae* is existing as a systemic infection in a plant.

Table 12. Preliminary tests of heuchera leaves using the semi-nested PCR reaction

Sample	**Cultivar**	**Description of tissue sampled and whether the plant was visibly infected**	**DNA amplification of *P. heucherae* obtained**
1.	Marmalade	Rust pustule (infected leaf)	Yes
2.	Marmalade	Area adjacent to a rust pustule (infected leaf)	Yes
3.	Marmalade	Mature leaf, no rust pustules (infected plant)	Yes
4.	Marmalade	Young leaf, no rust pustules (infected plant)	Yes
5.	Green Spice	Unknown status plant (no symptoms)	Yes
6.	Marmalade	Plant quarantined 2 years at the RHS (no symptoms)	No

Additional observations

Confirmation of rust infection on *Heucherella*

Visual assessment indicated that cv. Stoplight had fewer rust pustules per leaf than heuchera varieties Mahogany and Key Lime Pie in this planting. Plants of the tiarella variety Spring Symphony were not found to have rust pustules, despite their close proximity to other infected plants. Examples of leaves taken from the assessed plants are shown in Figure 6.

a

b

c

Figure 6. Heuchera rust on under and upper surface of leaves of plants from a garden group planting of heuchera, heucherella and tiarella. a) Heuchera cv. Key Lime Pie; b) Heuchera cv. Mahogany; c) Heucherella cv. Stoplight

Examination of the rust pustules found on *Heucherella* 'Stoplight' under 200x magnification confirmed the presence of low numbers of rust teliospores. DNA was extracted from a pustule on an infected leaf and the subsequent PCR amplification and DNA sequencing confirmed an identical internal transcribed spacer (ITS) DNA sequence to that of the rust *P. heucherae* found on heuchera plants.

Heucherella have thus been confirmed to be susceptible to *P. heuchera*, whereas *Tiarella* spp. have not yet been observed with rust infections.

Discussion

From the work under Objective 2.1, the current outbreak of rust on heuchera was identified as *P. heucherae* and confirmed through DNA sequencing of the ribosomal large sub unit (LSU) gene. This indicates that the rust outbreak is caused by the species that first originated in the UK in 2004 (Henricot *et al.,* 2007).

Although *P. heucherae* infection can result in rust pustules on the leaves or the stems, it is thought that there is likely to be a period of non-symptomatic infection prior to the development of the rust pustule (known as latent infection). This period of non-symptomatic infection means that heuchera plants may be arriving on a site with infection but the infection cannot be detected through visual examination of the plants. This uncertainty means that growers cannot be sure when infected heuchera plants are entering the supply chain.

One method to detect latent infection is to use a molecular test which detects the DNA of the rust fungus within the heuchera plant. The most common molecular tests are based on amplification of DNA (using a test called polymerase chain reaction or PCR).

When designing a molecular test it is important that the test is specific to the target pathogen, in this case *P. heucherae*. If the molecular test is not specific to *P. heucherae* then there is the possibility that false positive results may occur from other contaminating fungi in a sample. The amplification of the large subunit (LSU) and internal transcribed spacer (ITS) DNA sequences from rust pustules using existing broad range fungal PCR primers resulted in amplification of DNA from contaminating fungi present on the heuchera leaf. This caused a number of poor quality DNA sequences and the amplification of *Udeniomyces* and *Colletotrichum* DNA from two of the samples. This amplification of contaminating DNA demonstrated the need for a molecular test which had greater specificity *to P. heucherae*. A PCR test was therefore be designed with high specificity to *P. heucherae* DNA.

The PCR test for *P. heucherae* was designed against an online database of more than 33,000,000 fungal DNA sequences. This simulation for the molecular test indicated that a number of the newly designed primer pairs were theoretically specific to *P. heucherae*.

Molecular tests require high sensitivity to ensure that low levels of infection are detected and that infected plants are not incorrectly diagnosed as healthy plants (false negatives). The sensitivity of newly designed primers was tested against pure *P. heucherae* DNA and also a dilution of infected tissue and un-infected tissue. The molecular test was most sensitive when two rounds of DNA amplification was used, in a reaction known as a nested or semi-nested PCR.

The semi-nested PCR reaction with a first round of DNA amplification using primers 1F and 4R, and a second round of DNA amplification using primers 1F and 3R, was selected as the optimum reaction for the molecular test. This semi-nested PCR could detect as little as 10^{-15}g of rust DNA in a reaction, and as little as 0.001 % of infected tissue mixed with un-infected tissue. This high sensitivity will reduce the occurrence of false negatives occurring when the test is applied. The use of the primer pair 1F and 3R in the final round of DNA amplification for the semi-nested PCR ensured the specificity of the reaction to amplify only *P. heucherae* DNA.

The specificity of the semi-nested reaction was confirmed in the laboratory using DNA from 17 potential contaminating organisms. The semi-nested PCR reaction did not amplify a product from any of the non-target organisms, demonstrating the practical specificity of the newly designed molecular test to *P. heucherae*.

The newly designed molecular test was used to amplify DNA from the original mixed DNA samples from infected heuchera plants, and was shown to produce clean DNA products of *P. heucherae* from these mixed samples.

The newly designed molecular test was also used to test leaves from heuchera plants which were not showing visible symptoms, but which had visible infection symptoms elsewhere. The results from these preliminary PCR reactions clearly demonstrated that rust could be detected in leaf tissue before symptoms of infection were visible. This indicates that the molecular test can detect very low levels of infection in the plant. This is demonstrated by the positive amplification of *P. heucherae* DNA in a young leaf without pustules from an infected plant. This result may demonstrate the occurrence of localised symptomless infection points from spores landing on the leaf surface. It may also demonstrate that *P. heucherae* is existing as a systemic infection in a plant.

The molecular test was used in Objective 2.2 to detect whether latent infection is occurring in heuchera plants and to determine how long a latent *P. heucherae* infection can remain symptomless.

Once the molecular diagnostic techniques previously described had been developed then a new objective (Objective 4) was set out for this project in order to try to determine where in the heuchera production cycle *P. heucherae* could be first detected. Nurseries which had never had heuchera rust prior to 2010 believed that they might be receiving plants with latent rust as pustules were not visible on plants on arrival, but were seen later.

Objective 2.2: To apply the molecular test and collect and grow on heuchera plants to determine whether plants arriving at nurseries are infected by *P. heucherae*

Introduction

In order to seek to identify high risk entry points of heuchera rust in the supply chain, the molecular testing developed in Objective 2.1 could be utilised in the sampling of material over a year on nurseries dealing with plants at various stages of production. Material should be sampled as soon as possible after arrival at the nursery in order to avoid any post-delivery infection by any rust already present on the site.

By carrying out further observations for visible rust development after growing plants from each stage both under protection and away from any infection sources and outdoors, information could be gained on how long any latent infection might take to develop to produce symptoms on the plants and whether this might differ with growing conditions. This would allow investigation of the observations by growers surveyed that apparently healthy micro-propagated plants were developing symptoms about three to four weeks after delivery, or that plugs were showing symptoms a couple of months after potting up.

Information gained could provide indications on when to closely inspect plants, and when to apply fungicide control measures. In addition, whether to implement more stringent segregation of batches or to create quarantine areas.

Methods

Plant selection and suppliers

Heuchera cv. Marmalade is grown by numerous suppliers and was noted as one of the varieties frequently recorded with rust in the 2014 survey. Heuchera cv. Marmalade was therefore chosen for this study together with the seed-grown cultivar heuchera Palace Purple.

Visibly healthy plants were sampled at five points in the supply chain; seedlings, micro-propagated plant material, plugs/liners, 1L plants, and overwintered nursery stock. Plants were sampled from grower sites with a history of rust in previous years and the variety chosen has a known history of developing rust symptoms.

The supply chain for heuchera plants in the UK is complex, involving micro-propagators, plug growers and nurseries. An example schematic illustrating the complexity of the supply chain for a sample of 10 nurseries in the UK is shown in Figure 7. Suppliers for tissue culture plants are distributed globally and include Indonesia, Poland, Costa Rica, the Netherlands and the UK. A good working relationship was established with suppliers and nurseries across five separate supply chains and plants were sampled at defined growth stages (Table 13).

Table 13. Sampling schedule in 2015 for heuchera plants across the supply chains, using cv. Marmalade for all except the seed-sown plants which were cv. Palace Purple

Plant stage	Supply chain	Week number when sampled	Growth room and outdoors at RHS	No. of plants per growing condition	Total number of plants
Over-wintered	A	11	Yes	10	20
	B	12	Yes	8	16
Seed-grown	E	18	Yes	5	10
Tissue culture plants 2015	A	16	Yes	5	10
	B	17	Yes	5	10
	C	12	Yes	5	25
	D	20	Yes	5	25
Plugs 2015	A	20	Yes	10	20
	B	18	Yes	10	20
	C	16	Yes	10	20
	D	25	Yes	10	20
Finals 2015	A	*	*	*	*
	B	28	Yes	10	20
	C	35	Yes	10	20
	D	33	Yes	10	20

* Supplier was unable to provide plants

Sampling involved four micropropagators and one seed supplier who supplied four different nurseries. The supplied material was then collected from the nurseries as it grew into plugs and then 1 L container plants. Overwintered plants were from micropropagation in 2014, but were from the same sources at each nursery as the 2015 plants. Producers in supply chain ‘A’ provided tissue culture, plug, and overwintered plants, but were unable to supply final plants. Producers in supply chain ‘B’ provided tissue culture, plug, final and overwintered plants. Producers in supply chains ‘C’ and ‘D’ provided tissue culture, plug and final plants. The producer for supply chain ‘E’ provided seed-grown plug plants. Symptomless plants were tested with molecular diagnostics on arrival and grown further in a controlled environment

growth room away from potential infection sources (see details below and Figure 11) to enable development (and detection) of latent rust infections over several months to November 2015.

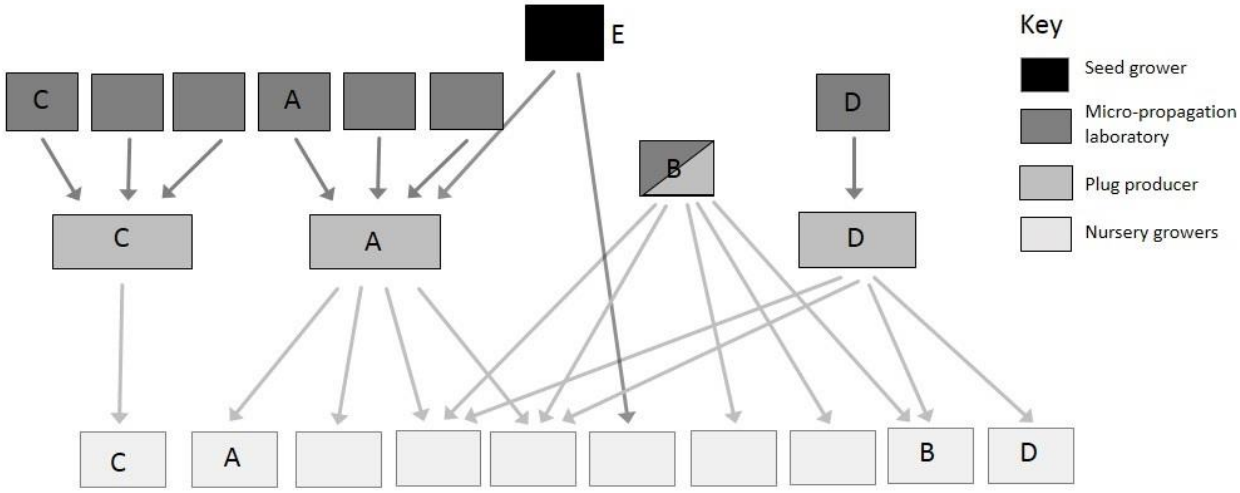


Figure 7. Schematic illustrating the complexity of the heuchera supply chain within the UK for a sample of 10 surveyed herbaceous nurseries. Nurseries A to E supplied material for testing for *P. heucherae* from each of the supply levels/plant stages shown in the key.

Half the plants from each supplier and each growth stage were randomly selected. Half the plants were grown in a controlled environment inside a polycarbonate growth room at the Field Research Facility at RHS Wisley. The plants held indoors were watered from the base three times a week with an automated drip irrigation system. Plant turgidity was maintained at all times. Indoor growth conditions were maintained at 18 °C day and 15 °C night, 12 hour day with additional lighting as required to reach 2000 Lux, and 60 % relative humidity. The plants were arranged in a balanced random block design designed using the statistical software GenStat 11th Edition (see Appendix 3 for a summary of the random block design). The other half of the plants were arranged in the same random block design outdoors on woven ground-cover matting (Figure 11) with daily overhead irrigation to supplement rainfall. Daily temperature and rainfall weather data are presented in Appendix 4.

Initial sampling for molecular testing

On arrival all plants were inspected for visible rust symptoms. The tissue culture plants were too small to sample three leaves without destroying the plant (see Figure 7 for examples of tissue culture plants). Therefore five whole tissue culture plants were destructively sampled and frozen at -80 °C for DNA extraction and PCR. A single leaf was taken from each of 20 further tissue culture plants, pooled into batches of five leaves per sample and processed for DNA extraction and PCR. Ten of the tissue culture plants were then rooted and grown on for continued observation (as described in the following section). This procedure was repeated for the small seed-grown plants. For the larger plug plants, finals and overwintered plants, three leaf discs from three leaves across each plant were pooled and frozen at -80 °C for DNA extraction (Figure 8).

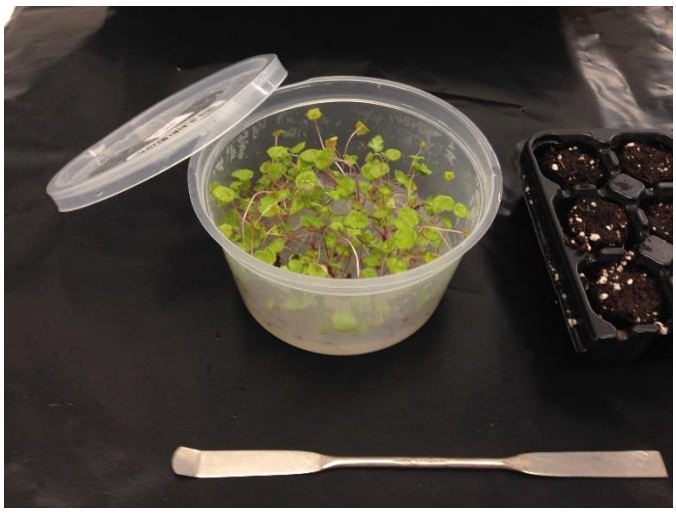


Figure 7. Small tissue-culture heuchera plants

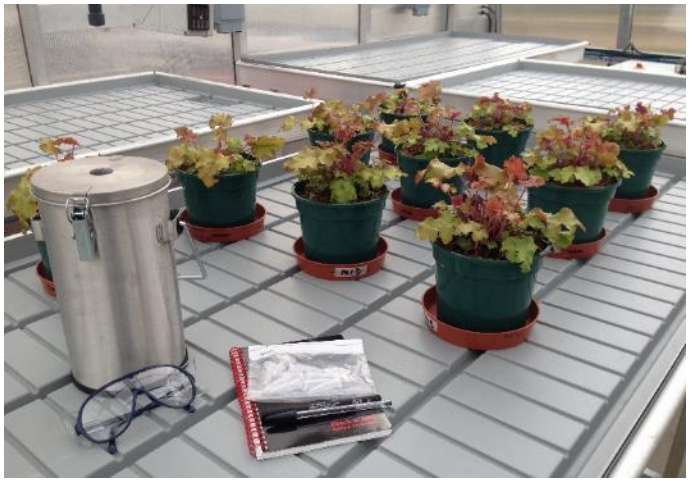


Figure 8. Initial sampling of overwintered heuchera for molecular testing

The samples were processed for molecular testing. Each sample was ground to a fine powder using a micro-pestle and liquid nitrogen. The powdered plant material from each sample was then extracted using a QIAGEN DNeasy plant mini kit. PCR reactions were performed using 1 x Bioline Biomix in a 25 µl reaction with 1 µl template DNA and 35 amplification cycles. A semi-nested PCR reaction was used with primers 1F and 4R in the first amplification and primers 1F and 3R in the second amplification. The second round of amplification included the newly designed primer pair 'Heuchera ITS Forward' and 'Heuchera ITS Reverse', to amplify a 170 bp control product from the ITS region of the heuchera plant. The amplified products were run on a 1.5% agarose gel at 5V/cm and viewed under ultra-violet light. An example gel image for PCR reactions is shown in Figure 9.

Amplification of a DNA product of size 430 bp indicated a plant was infected with *P. heucherae*.

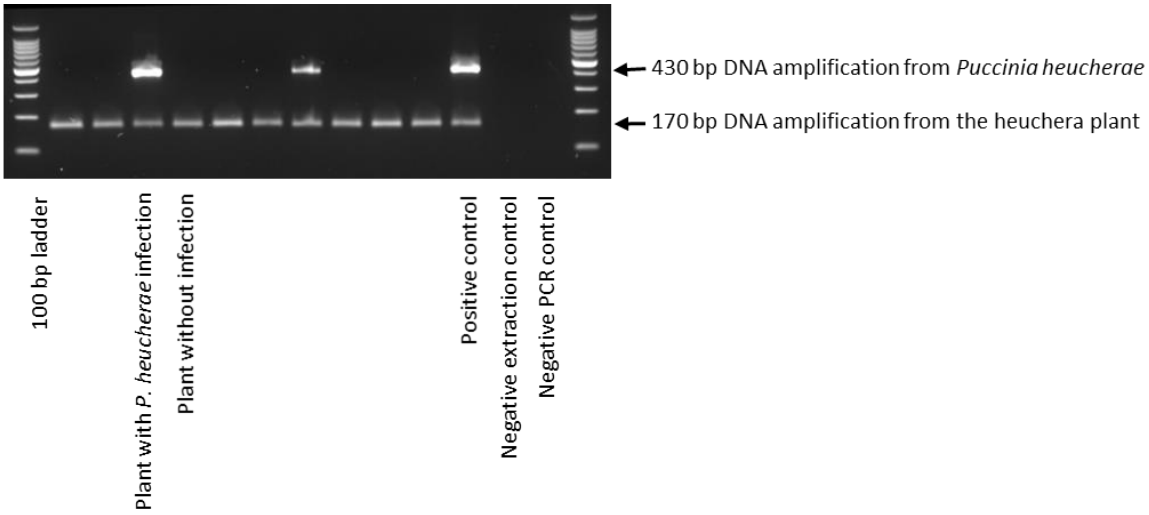


Figure 9. Gel image showing amplified DNA products following PCR using the newly designed semi-nested *Puccinia heucherae* primers and the control PCR primers for amplification of heuchera plant DNA.

Continued growth and monitoring of plants for rust infections

To support the molecular test, the heuchera plants were grown further and monitored visually each week for rust infection.

The tissue culture plants were initially rooted in coir-based modules and subsequently potted into 9 cm (Figure 10) and 1 L pots. The plug plants were initially potted into 9 cm pots and then re-potted into 1 L pots. The potting mix used was a mix of two parts John Innes No. 2, two parts J Arthur Bowers Multipurpose and 1 part Vermiculite. No further feed was applied through the growth season.

Plants were visually inspected on a weekly basis for the development of rust symptoms. If rust was detected, the plant was isolated from other plants at the first development of symptoms, i.e. before the release of spores (to eliminate secondary infection). Rust infections were confirmed through microscopic analysis of the pustule for the presence of teliospores. Plants were inspected weekly for development of infection through to 1st November 2015.

Figure 10. Tissue culture plants rooted in coir based modules and potted into 9 cm pots

a

b

Figure 11. Heuchera plants for continued observation at the RHS Field Research Facility October 2015. a) plants in controlled environment greenhouse, b) plants held outside.

Testing for systemic rust in naturally infected plants in tissue not showing symptoms

The PCR molecular test was utilised in an additional primary investigation with a small number of plants that had been collected from nurseries in 2014 with visible rust pustules and that still had visible old pustules after a year. Plants of cv. Ruby Bells, cv. Chocolate Ruffles and cv. Rio had shown extensive fresh pustule development in 2014 and were overwintered outdoors at ADAS Boxworth without cutting back and grown through 2015 under overhead irrigation. By December 2015 no new pustules had developed. One plant of each cultivar was delivered to RHS Wisley for molecular testing.

Tissue was taken from three leaves per plant to obtain material both without visible symptoms, and directly from tissue with a pustule. The inside of the plant crown of cv. Ruby Bells was also subjected to the PCR test.

Results

Neither the molecular test nor the continued monitoring of plants detected the presence of rust in any of the tissue culture plants, the seed grown plants, the plugs or the finals from any supplier (Table 14).

Two asymptomatic over-wintered plants from a supplier in supply chain 'B' tested positive for rust with the molecular test on arrival in March 2015. Neither of these plants subsequently developed visible rust symptoms within the nine months of observations indoors and outdoors.

Two different plants from supply chain 'B' subsequently developed a rust pustule on a single leaf four weeks and five weeks post arrival. PCR tests on samples from around the pustule on the same leaf were negative. One of these symptomatic plants was from the outdoor set of plants, and the other was from the indoor set.

Table 14. Summary of results from molecular testing and continued observations of plants at RHS Wisley to 1 November 2015. All except seed-sown heuchera were cv. Marmalade

Plant stage	**Pictures of plants at time of PCR testing**	**Detection of latent and visible rust**
Over-wintered heuchera 2014 - 2015		Two symptomless mature plants from supply chain 'B' tested positive for rust infection using the molecular test when sampled in Weeks 11 and 12. Neither subsequently developed rust symptoms. Two further symptomless plants from supply chain 'B' developed rust symptoms in Weeks 16 and 17, one held outdoors the other indoors. No rust infected plants were detected in the plants from supply chain 'A' neither by molecular test nor continued observation of plants.
Plant stage	**Pictures of plants at time of PCR testing**	**Detection of latent and visible rust**

Tissue culture 2015		No rust infected plants were detected from any supply chain ‘A’ ‘B’ ‘C’ or ‘D’ micropropagated plants either by molecular test on arrival in Weeks 12 and 17 or by continued observations.
Seed-grown 2015		No rust infected cv. Palace Purple plants were detected in supply chain ‘E’ by the molecular test in Week 18, nor after continued observation.
Plugs 2015		No rust infected plants were detected from either supply chain ‘A’ ‘B’ ‘C’ or ‘D’ by the molecular test in Weeks 16 and 25, nor after continued observation.
Plant stage	**Pictures of plants at time of PCR testing**	**Detection of latent and visible rust**

1 L pots 2015		No rust infected plants were detected from supply chain ‘B’ ‘C’ or ‘D’ when tested with the molecular test in Weeks 28 and 35, nor after continued observation.

Testing for rust in naturally infected overwintered plants in tissue not showing symptoms

Three leaves collected from each of three plants (cv. Ruby Bells, cv. Chocolate Ruffles and cv. Rio) which had pustules remaining from 2014 (but no further pustule development during growth outside during 2015) tested positive for *P. heucherae* using the PCR test when pustules were sampled. However, when leaves on the same plants but without pustules were tested these were negative.

Only the cv. Ruby Bells had a suitable root/crown area for sampling as the other two had become damaged by vine weevil larvae (Figure 12). The sample tested negative for *P. heucherae* using the PCR based molecular test.

Systemic rust infection through the plant (potentially from the leaves via the petioles into the crown and then back up into new leaves) was not shown to occur in the plants tested.

In 2015 the remaining leaves had flat brown rust pustules formed of teliospores. Several leaves had old necrotic spots where the teliospores had been. When pustules were examined under the microscope no basidiospore production was able to be seen from the teliospores.

Figure 17. 20 month old rust infected shoot of heuchera cv. Ruby Bells, showing the stem base/upper root area tested for the presence of *P. heucherae*

Discussion

Molecular tests and continued monitoring of tissue culture plants, seed grown plants, plug plants and finals have indicated these plants were not infected with rust. In this study infection was only detected in the overwintered plants (2014-2015). The detection of rust infection in the overwintered plants equates to infection in 4 out of a total of 36 (approximately 10 %) of the overwintered plants which were non-symptomatic on arrival. Overwintered plants may therefore be a source of infection in the spring and should be monitored closely or kept isolated from newly-sourced heuchera plants.

The development of rust pustules on two overwintered plants five weeks after delivery and being held in a greenhouse at RHS Wisley (after previously testing negative using the molecular test), indicates that these infections were not systemic and had developed from localised infection points. Further evidence of the lack of systemic movement of the pathogen in the plant was obtained from the samples of naturally infected plants overwintered outdoors, followed by a further summer outdoors at ADAS Boxworth. If infections are occurring as localised infection points and are not systemic in the entire plant then it would be possible to control infections by cutting back old leaves to break the infection cycle. If growers must overwinter heuchera plants, cutting back old leaves to the crown may therefore be beneficial in reducing the occurrence of fresh infective pustules in the spring. This also helps to thin the canopy and to reduce the retention of water between the leaves that can favour rust infection.

Detection of two infected plants using the molecular test and the subsequent lack of development of visible infection on these plants indicates that not all infections lead to visible symptoms. Unfortunately it is not possible to distinguish between DNA from active and dead cells using a molecular test. This result may therefore be caused by a long-term latent infection, or an infection which has been successfully controlled by application of a fungicide and is no longer active.

Unfortunately for this study the year 2015 appears to have had a very low incidence of heuchera rust. Numerous growers spoken to in 2015 have reported no rust or very low levels of rust, although the situation is hard to judge as most now have a protectant fungicide spray programme and many have ceased growing the varieties that they have previously had noticeable rust on (R. Robinson and E. Wedgwood pers. comm.). The lack of detection or development of rust in the tissue culture, seed grown, plugs and final plants has meant it is not possible to draw conclusions from this experiment as to the influence of growth conditions and watering regimes on the subsequent development of rust symptoms.

Similarly, whilst no infection was detected on the tissue culture plants or plugs this year, the lack of infection in the finals limits the scope of the conclusions which can be made. However, we would suspect that tissue cultures provide a clean plant source and this is affirmed in the results of this year. Further study would be required in a year with a high incidence of rust to confirm that this is generally true.

From the small sample of plants tested it would appear that there is not systemic movement of the pathogen into other leaves or the plant crown following aerial infection of a leaf. It would be expected that the DNA would still be detected even if for some reason the pathogen was killed within the plant over the 12 months since fresh infection was last seen. The cutting back of plants with infection already practiced by some growers, therefore has the benefit of reducing further production of inoculum, but it is probably not needed to stop the movement of infection within the plant. If infested leaves are cut off by growers it is important that this is done under dry conditions otherwise this could allow the movement of infested leaf material onto uninfested leaves. Further sample testing would be worthwhile to confirm that infection is not systemic.

Objective 3: To determine the mode of infection by *P. heucherae* and the environmental conditions required for the infection of heuchera plants

Introduction

Many rusts have a life cycle that involves the production of pustules of more than one spore type during a calendar year, with a number of species having an alternate host on which they spend a part of their lives. One of the hosts is usually used as a means of overwinter survival and so pustules of thick-walled spores called teliospores (or teleutospores) are produced. Dispersal spores called basidiospores are produced from out of the body of the teliospores, which may or may not themselves disperse and germinate (according to the species). In *Puccinia xanthii* (cocklebur *Xanthium* spp. rust) teliospores germinate while still attached to telia when exposed to high relative humidities. *Puccinia poarum* produces telia on *Poa* spp. grasses, but has pustules of a second spore stage on *Tusillago* sp. (coltsfoot). However, *P. heucherae* is relatively unusual in that only the teliospore stage is known.

Another *Puccinia* species *P. horiana*, the cause of white rust disease of chrysanthemum, has only teliospores and associated basidiospores. In this species basidiospore release, germination and infection can take as little as 5 hours at optimum relative humidity (96%) and temperature (between 17–24°C). A significant amount of research has been carried out on *P. horiana* including varietal susceptibility (Firman and Martin, 2008) and a review of the biology, aetiology and epidemiology is reviewed in relation to current environmental, cultural and chemical methods for its control was carried out (Whipps, 2008). Breeders of chrysanthemums, Yoder, have provided a bulletin on control measures for chrysanthemum growers based on research findings (Rizvi, 2011). The germination of *Puccinia xanthii* teliospores was shown to be stimulated by either continuous darkness or 12 hours darkness Basidiospore production by P*. xanthii* is significantly greater the longer the period of high humidity, being greatest at 20°C, and inhibited by light regimes beginning with a dark period (Morin *et al*., 1992).

It is possible that *P. heucherae* could share some or all of these infection requirements, and so research to determine these could lead to advice for heuchera growers such as not watering prior to darkness or targeting any fungicide applications to within two hours after a period of leaf wetness before germ tubes enter the host.

There were two main components of Objective 3:

- To determine by artificial inoculation using spreader plants and pustule contact whether infection takes place via basidiospore discharge and/or direct teliospore infection (preliminary inoculation experiment).
- To determine the period of leaf wetness required for *P. heucherae* infection under night and day lighting conditions (main inoculation experiment).

Methods

For details of the experiments included in Objective 3, please refer to the Annual Report for 2015. The preliminary experiment exposed healthy heuchera to either direct pustule contact or to confinement with plants with pustules able to produce basidiospores. In the main experiment, plants were exposed to both direct pustule contact and the possibility of basidiospore discharge from infector plants during periods of two, four and eight hours of leaf wetness, both during dark and lit periods of 12 hours.

Observations to record pustule development were made from 2014 to towards the end of 2015 when mature leaves were sampled in order to determine if latent infection was present.

Results

No rust infection was confirmed in either experiment, either by the presence of pustules or by the limited sampling of leaves and then using PCR testing for latent *P. heucherae* infection.

Discussion

It is possible that no infection developed because the environmental conditions, which would have been suitable for infection in other rust/host interactions, are not suitable for *P. heucherae*. However until further work is carried out to determine what the optimum conditions are and unfavourable conditions confirmed then caution should be exercised. Leaf wetness and/or high humidity are likely to be required for germinating spores, but more information is needed on basidiospore dispersal conditions. Molecular tests on samples did not show the presence of latent rust in mature leaves from any of the treatments. However, not all leaves present at the time of inoculation were able to be sampled (molecular testing is done with very small amounts of tissue), and as tests under Objective 2 have indicated that heuchera rust is unlikely to be systemic, it is possible that infected leaves could have been missed.

Teliospores were observed to be fixed within the pustule, not loose and powdery and so for direct germination (rather than basidiospore discharge) to occur there would need to be close contact between the pustule and new host tissue. New infection on young leaves of plants with pustules on old leaves is most likely to originate from basidiospores released from teliospores, perhaps facilitated by water splash. It is however, still possible that there is a yet

undiscovered alternate host for *P. heucherae* that releases another spore type that blows in to infect the new heuchera leaves.

General Conclusions

Surveys of growers who responded via the AHDB showed that there was knowledge that leaf wetness and humid conditions could encourage the development of rusts, however all were using overhead irrigation (including those who reported no rust). In the USA, the method of allowing heuchera to flag before watering and so thicken the cuticle against rust is sometimes employed.

It was not possible to determine any patterns of rust source from the grower survey information. Most varieties (with and without rust commonly reported) were bred by Terra Nova in the USA who said that they now avoid breeding yellow leaved varieties because of rust susceptibility. Lighter leaved varieties were popular and reported by some UK growers to have rust, however, except for Peach Flambé, they were more often reported to be rust free. Darker varieties such as Palace Purple, Berrie Smoothie and Plum Pudding were also popular but without any rust seen.

In studies in 2014, active pustule production was not observed during the summer on outdoor untreated plants. Most growers who had seen rust on their nurseries in previous years were now using preventative fungicide programmes and it was likely that low heuchera rust incidence in 2014 was as a result of this. It was conjectured that *Puccinia horiana* (white rust of chrysanthemum), which also only produces teliospores, may have a similar life cycle to *P. heucherae,* which was confirmed by molecular tests in eleven varieties sampled in 2014. These spores do not brush off the leaves to infect, but produce air-borne basidiospores at a relatively low optimum temperature of 17°C (such as commonly found in spring and autumn). The teliospores remain on leaves throughout the life of the leaf (which stay on the plant all year even through frosts), although in some instances the whole pustule drops out to leave a hole.

Heuchera rust was observed in 2014 to produce only teliospore cluster pustules on heuchera, but it was not possible to obtain visible infection under experimental conditions. Many growers reported overwintered heuchera to be frequently affected by rust and in the course of 2014 reports only came from these older plants with dense canopies.

A molecular test (nested PCR) was developed for *P. heucherae* which confirmed that this was the species affecting heuchera and heucherella in the UK. It also proved to be sensitive

enough to detect symptomless infection by *P. heucherae* (although such molecular tests are unable to distinguish whether the DNA belongs to a dead or alive organism). Using this test on symptomless leaves from a plant with infection visible elsewhere indicated that infection is not systemic within a plant, nor was systemic infection found in the crown of plants that had become infected a year previously. Therefore, although it is feasible for growers to use molecular testing of material, it would require destructive sampling of micro-propagated plants arriving in agar dishes, and in larger plants the test would only apply to the leaf sampled.

Molecular testing did not find any evidence of latent *P. heucherae* in young plants of cv. Palace Purple grown from seed in 2015. When samples of cv. Marmalade (identified as a variety in which rust had been seen by a number of growers surveyed) were taken for molecular testing from several sources along the supply chain the only detection of *P. heucherae* was in plants that were sampled in spring 2015 after overwintering. Of the material produced in 2015, no rust was detected in micro-propagated stage III material arriving on nurseries, nor on plug plants entering nurseries, nor on 1 L pots destined for retail. None of the material produced in 2015 subsequently developed symptoms, whether held outside or under protection. However, some of the plants overwintered into 2015 developed symptoms over five weeks in late April and it is concluded that plants of all stages, but in particular overwintered, should remain under observation for the development of visible rust for at least a month after delivery.

Knowledge and Technology Transfer

1. AHDB Herbaceous Perennial Technical Discussion Group Winter Meeting. London. 11 February 2015. Powerpoint presentation.
2. “A pathogen in hiding”. Article in AHDB Grower, October 2015 pp. 4-5
3. AHDB Horticulture Herbaceous Perennial Technical Discussion Group Meeting, 16 February 2016 R. Robinson presented on the molecular detection of heuchera rust.
4. Poster produced for the AHDB Horticulture Pest, Disease and Weed Control Conference “Devising sustainable solutions”. 23 February 2016. Stoneleigh, Warwickshire.
5. A factsheet will be produced for growers from the outputs of the project.

Glossary

Information from Alexopoulus (1962).

Basidiospore: A germ tube (promycelium) is produced from one or both of the teleutospore cells into which the nucleus migrates to divide to produce four nuclei. These are moved out through four separate stalks. A basidiospore is formed on each of these stalks and then forcibly ejected.

Pustule: A cluster of spores which break through the leaf tissue at the site of rust infection, usually forming a raised area.

Teliospore or Teleutospore: A thick-walled resting spore, notably found in the rusts and smuts

Telium (plural Telia): A group of binucleate cells which produce clusters of teliospores. There may be several telia in a pustule.

References

Alaei H., Baeyen S., Maes M., Höfte M. and Heungens K., 2009. Molecular detection of *Puccinia horiana* in *Chrysanthemum* × *morifolium* through conventional and real-time PCR. Journal of Microbiological Methods, 76(2):136-145.

Alexopoulus, C.J. (1962). Introductory Mycology. Second Edition. Wiley. 613 pp.

Brough, W. (2013). Heuchera rust - where next. AHDB Herbaceous Perennials Technical Discussion Group. Foliar Diseases of Herbaceous Plants. 21 February 2013.

Farr D.F., Bills G.F., Chamuris G.P. and Rossman A.Y. (1995) Fungi on plants and plant products in the United States. APS press, St Paul, Minnesota, USA.

Farr D.F., Rossman, A.Y., Palm, M.E and McCray E.B. (no date). Fungal Databases, Systematic Botany & Mycology Laboratory, ARS, USDA. Retrieved January 13, 2006, from http://nt.ars-grin.gov/fungaldatabases/

Firman, I. D. and Martin, P.H. (1968). Article: White rust of chrysanthemums. Annals of Applied Biology 62: 429-442.

Gardes M. and Bruns T.D. (1993). ITS primers with enhanced specificity to basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular Ecology 2, 113-118. doi: 10.1111/j.1365-294X.1993.tb00005.x

Henderson D.M. (2000). Checklist of the rust fungi of the British Isles. British Mycological Society, Richmond

Henricot B., Denton G. and Lane C. (2007). First report of *Puccinia heucherae* on *Heuchera* spp. in the UK. Plant Pathology 56: 352.

Kropp, B.R., Hansen, D., Flint, K.M., Thomson, S.V. (1996). Artificial inoculation and colonization of Dyer's Woad (*Isatis tinctoria*) by the systemic rust fungus *Puccinia thlaspeos*. *Biological Control*, 86 (8), 891-896.

Maier W., Begerow D., Weiß M. and Oberwinkler F. (2003). Phylogeny of the rust fungi: an approach using nuclear large subunit ribosomal DNA sequences. Canadian Journal of Botany 81: 12-23

Morin, L., Brown, J.F. and Auld B.A. (1992). Effects of environmental factors on teliospore germination, basidiospore formation, and infection of *Xanthium occidentale* by *Puccinia xanthii*. Phytopathology, 82 (12), 1443-1447.

Pavlich, C. (2013). Newest developments in breeding rust-resistant heucherella. http://www.newplantsandflowers.com on-line article of 1 October 2013 accessed 26.01.2015

RHS Advice profile. Heuchera rust.
https://apps.rhs.org.uk/advicesearch/Profile.aspx?pid=774

Rizvi, S.A. (2011). Yoder Mums, Syngenta flowers. Chrysanthemum White Rust Bulletin. 6 pp. http://nysipm.cornell.edu/pest_alert/chrys_white_rust/CWR_BulletinYoder.pdf

Savile D.B.O. (1954). Taxonomy, phylogeny, host relationship, and phytogeography of the microcyclic rusts of Saxifragaceae. Canadian Journal of Botany 32: 400-425.

Terra Nova Nurseries FAQ.
http://www.terranovanurseries.com/gardeners/heucheracherrycola-p-292.html

Scrace, J. (2000). Factsheet 23/00. Bedding plants. Project No. PC 175. Horticultural Development Council.

Scrace, J. (2012). Rust diseases of herbaceous plants with specific reference to heuchera rust AHDB Herbaceous Perennials Technical Discussion Group. 18 July 2012.

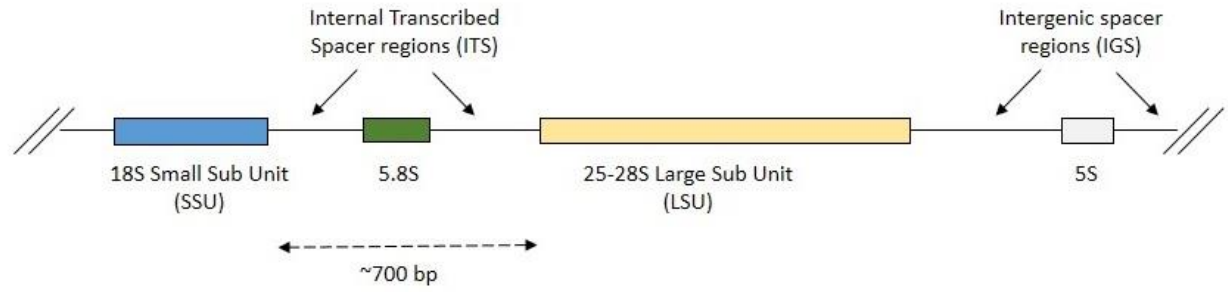
Ward, N. A., Schneider, R. W. and Robertson C. L. 2012. Documentation of an extended latent infection period by *Phakopsora pachyrhizi*, the soybean rust pathogen. Online. Plant Health Progress doi:10.1094/PHP-2012-0321-01-RS.

Wilson M. and Henderson D.M., 1966. *British Rust Fungi*. Cambridge, UK: Cambridge University Press.

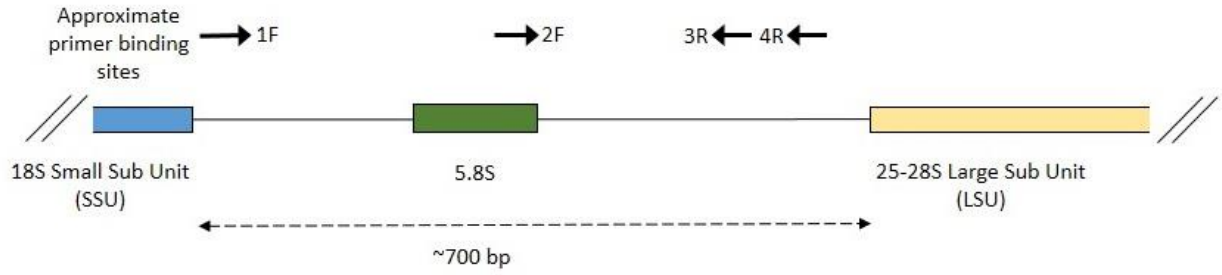
Whipps, J. M. (1993). Article: A review of white rust (*Puccinia horiana* Henn.) disease on chrysanthemum and the potential for its biological control with *Verticillium lecanii* (Zimm.) Viégas. Annals of Applied Biology, 122, 429-442.

Appendices

Appendix 1. Structure of the fungal ribosomal RNA gene operon and the approximate binding positions of the newly designed *Puccinia* primers within the ITS region



Primer binding sites in the ITS regions



Appendix 2.

BLAST searches for each primer against the NCBI fungal DNA database demonstrated that the newly designed primers are specific to rust fungi and highly optimised for amplification of the genus *Puccinia.*

Successful PCR amplification requires both the forward and reverse primer in the reaction to bind to the target DNA in order to amplify a product. Only contaminating organisms with DNA sequences which allow binding of both the forward and reverse primer within a PCR amplification pose a risk for non-target DNA amplification. The specificity of the PCR reaction is therefore improved when the primers are paired into their forward and reverse components. The primer pairs 1F and 2R, 1F and 3R and 2F and 4R do not bind to any common non-target organisms so are theoretically specific to *Puccinia heucherae*. The primer pairs 1F and 4R, and 2F and 3R both bind to one non-target *Puccinia* species.

ITS primers specificity as indicated by a BLAST search of the NCBI DNA database (GenBank)

Primer	Sequence (5' to 3')	BLAST search for specificity Non-target matches
1F	ATTGTGGCTCGACCCCTTTTA	*Puccinia* (8 spp.) and *Uromyces* (1 spp.)
2F	GCATCTTGCACCTTTTGGTATT	*Puccinia* (4 spp.)
3F	GTGGATGTTGAGTGTTGCTGT	*Puccinia* spp. (14 spp.) and *Gymnosporangium* (4 spp.)
2R	ACCAAAAGGTGCAAGATGCG	*Puccinia* spp. (6 spp.)
3R	ACAGCAACACTCAACATCCAC	*Puccinia* spp. (14 spp.) and *Gymnosporangium* (4 spp.)
4R	AGTATTTGACTACTTTCCTTAATGC	*Puccinia* (9 spp.)

ITS primer pair specificity as indicated by a BLAST search of the NCBI DNA database (GenBank)

PCR Primer pair	Theoretical amplification of non-target DNA
1F and 2R	None
1F and 3R	None
1F and 4R	*Puccinia coronata*
2F and 3R	*Puccinia graminis*
2F and 4R	None

Appendix 3. Layout of heuchera plants at the RHS Field Research Facility

Bench 1

B - P - 1	D - F - 1	C - TC - 1	C - P - 1	B - F - 1	C - F - 1	A - P - 2
D - TC - 1	B - P - 2	A - TC - 1	E - S - 1	C - P - 2	B - TC - 1	A - P - 1
A - F - 1	C - F - 2	D - F - 2	A - F - 2	D - P - 1	B - F - 2	D - P - 2

Bench 2

C - P - 4	D - P - 4	A - P - 4	B - P - 3	B - P - 4	C - F - 3	D - F - 3
A - P - 3	D - F - 4	B - F - 3	E - S - 2	B - TC - 2	C - F - 4	A - F - 4
A - F - 3	C - TC - 2	B - F - 4	D - TC - 2	D - P - 3	A - TC - 2	C - P - 3

Bench 3

C - F - 5	C - P - 6	D - P - 5	B - P - 5	E - S - 3	B - F - 5	B - TC - 3
A - F - 5	A - F - 6	D - TC - 3	D - F - 5	B - P - 6	A - P - 6	C - P - 5
C - F - 6	D - F - 6	A - TC - 3	D - P - 6	B - F - 6	A - P - 5	C - TC - 3

Bench 4

D - F - 8	C - P - 7	D - P - 8	E - S - 4	B - P - 8	B - F - 8	D - TC - 4
A - F - 8	C - TC - 4	B - F - 7	B - P - 7	D - F - 7	A - P - 8	A - TC - 4
A - F - 7	C - F - 7	D - P - 7	C - F - 8	B - TC - 4	C - P - 8	A - P - 7

Bench 5

D - P - 10	D - TC - 5	A - F - 10	C - F - 10	E - S - 5	D - F - 10	B - F - 9
B - F - 10	B - P - 10	D - F - 9	A - P - 9	A - F - 9	C - TC - 5	B - P - 9
C - P - 10	C - P - 9	C - F - 9	A - P - 10	B - TC - 5	D - P - 9	A - TC - 5

Bench 6

A - OW - 4	A - OW - 8	A - OW - 5	B - OW - 5	A - TC - 6	B - OW - 4	B - OW - 7
B - OW - 1	A - OW - 10	A - OW - 1	B - OW - 6	A - OW - 3	D - TC - 6	A - OW - 9
A - OW - 7	B - OW - 8	A - OW - 6	C - TC - 6	A - OW - 2	B - TC - 6	B - OW - 3

Key:

Supply chain A-E ----- Growth stage ------ Plant number

Growth stages: TC Tissue culture, P Plug, F Final, OW Overwintered

Appendix 3.

Daily rainfall (mm) and Maximum and minimum temperatures (°C) experienced by outdoor heuchera kept under observation for rust at RHS Wisley January – November 2015