Project Title:	Red Beet: Further Elucidation of the Cause, Epidemiology and Control of Root Malformation Disorder (RMD)
Project Number:	FV 226d
Project Leader:	Dr G M McPherson MBPR (Hort.) Technical Director Crop Protection Services Stockbridge Technology Centre Cawood, Selby North Yorkshire YO8 3TZ
Report:	Final Report, April 2006
Location:	STC Ltd and grower crop trials in South Yorkshire
Project Co-ordinator:	Mr G Smith R Smith & Sons South Carr Farm Westwoodside Doncaster DN9 2EW
Date Commenced:	May 2005
Completion date:	November 2005
Key Words:	Red beet, beetroot, root distortion, malformation, RMD, downy mildew, *Peronospora farinosa*, *Rhizoctonia*, *Pythium* spp., disease control, fungicides, crop safety, efficacy, disease.

The results and conclusions in this report are based on a series of laboratory experiments and a field trial on a commercial crop of red beet. The conditions under which the experiments were carried out and the results generated have been reported with detail and 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 to be used as the basis for commercial product recommendations.

It should also be noted that many of the products tested in this work are experimental in nature and under no circumstances should they be used commercially. If anyone is in doubt regarding the current approval status of a particular product they should either consult the manufacturer, check the status on an approved pesticide database, or take independent advice from a BASIS qualified adviser.

AUTHENTICATION

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

Signature……..……

Ms C Lambourne
Project Manager
Stockbridge Technology Centre

Date……

Signature…….

Dr N Boonham
Central Science Laboratory

Date ……..

Report authorised by……..

Dr G M McPherson MBPR (Hort.)
Director – Plant Pathology
Stockbridge Technology Centre

Date…….

Stockbridge Technology Centre Ltd
Cawood, Selby
North Yorkshire
YO8 3TZ

Tel. 01757 268275
Fax. 01757 268996

CONTENTS

FV 226d : GROWER SUMMARY

Red Beet : Further Elucidation of the Cause, Epidemiology and Control of Root Malformation Disorder (RMD)

Headlines

- Low levels of downy mildew and RMD were observed in both trial sites and commercial crops in 2005.
- Therefore the trial did not gather any new data as treatment effects could not be measured.
- Downy mildew was detected in seed lots in 2005, although it cannot be determined whether the disease was viable.

Background and Expected Deliverables

Previous work on the root malformation disorder (RMD) in red beet has eliminated various possible causes for the problem and has recently focused on the possibility that the distortion is caused by a systemic infection with the downy mildew pathogen *Peronospora farinosa* f.sp. *betae*. For more comprehensive information on previous work see previous HDC reports for FV 226, FV 226a-c.

A range of fungicides with activity against this important beet pathogen have been secured, primarily via the HDC-funded SOLA programme and this has potentially provided growers with a short-term solution to the problem. Since a range of oomycete fungicides were approved and made available to the beet industry neither downy mildew nor RMD have been problematic in commercial crops. However, RMD has been sporadic in its occurrence previously and its absence in the last 3 seasons does not necessarily provide a categorical assurance that a spray programme comprising oomycete fungicides will necessarily prevent a re-occurrence of the RMD problem that caused such economic damage in 1998 & 2002. In addition, assuming the downy mildew hypothesis is correct, it is still necessary to determine how and when the infection occurs and when fungicides need to be applied to maintain effective season-long protection against RMD.

For a final confirmation of cause and effect between downy mildew and RMD it is necessary to demonstrate Koch's postulates. However, this is not necessarily straightforward as the fungus is an obligate (non-culturable) pathogen and cannot be raised in culture or isolated onto artificial agar media from affected roots. To date, this has been countered by using a modern molecular PCR (Taqman) assay to demonstrate the presence of DNA of the downy mildew pathogen in RMD affected beet. At the same time, and in contrast to this result, similar tests on visibly healthy beet roots have demonstrated an absence of *Peronospora* DNA internally. However, without appropriate studies we cannot be absolutely certain at this stage that the PCR test isn't cross-reacting to other pathogenic or saprophytic fungi in the affected roots. Therefore, at the present time, the molecular assay cannot be used alone as a substitute for artificial inoculation studies to prove Koch's postulates.

The purpose of the work conducted in 2005 therefore was to:-

(i) undertake a replicated fungicide timing trial in a high risk unsprayed beet crop (in the hope that RMD would be problematic)
(ii) Undertake cross-reaction studies with the molecular PCR test to see if positive reactions were gained against other fungi commonly found on red beet, particularly other oomycetes
(iii) To validate and use the molecular PCR test to check seed-lots of commercial red beet for the possibility of seed-borne d. mildew.

Summary of the Project (2005) and Main Conclusions

The work undertaken in 2005 was scaled down to a single replicated fungicide timing trial at South Carr Farm in Westwoodside, South Yorkshire. By August it became clear from our own assessments and information from red beet growers that there had been no d. mildew infections observed on crops in the area. However, it remains unclear whether the absence of RMD symptoms was due to a natural disease cycle, whether the warm, dry spring reduced the risk of d. mildew or whether the increased use of seed treatments and fungicides aimed at d. mildew control prevented its development in 2005.
.
As in previous years, molecular testing of red beet samples was carried out by CSL. The lack of observed d. mildew and RMD symptoms in the roots of red beet crops in 2005 prevented effective crop monitoring and 'tagging' during the season and therefore molecular testing of such samples was not possible.

A total of six batches of seed were tested using the TaqMan methodology for the presence of *P. farinosa* DNA. Based on the results it indicated that *P. farinosa* DNA was present at varying levels in all six of the beet seed-lots tested, although the quantity of DNA detected varied widely within each seed lot. However there is still the possibility of an anomalous cross-reaction in the test; hence the need for further validation, so firm conclusions cannot be drawn at this stage.

We still cannot confirm with 100% certainty that RMD is caused by a systemic infection by downy mildew (*P. farinosa* f.sp. *betae*), though, in the absence of other likely causes, this continues to be the most likely possibility.

Financial Benefits

The financial benefits from this study cannot be fully determined until such time that the industry has the confidence that they can effectively control the problem with the application of oomycete or other fungicides.

Action Points for Growers

- Growers need to maintain their vigilance, avoid complacency and continue to be aware of the continued risk from RMD in red beet and the potential economic significance should it re-occur.
- Beet crops should be monitored from emergence onwards for the first signs of downy mildew, root malformation or other possible symptoms that may be associated with the problem.
- Where downy mildew or RMD symptoms are found growers are encouraged to alert members of the research team and/or the chairman of the Red Beet Technology Group so that it can be followed up and appropriate tests conducted.
- Until such time that the primary cause of RMD can be fully elucidated and high risk periods identified (i.e. environmental parameters most conducive to RMD development) growers are encouraged to develop and maintain an effective preventative disease control strategy using a range of oomycete fungicides, including seed treatment, where possible.
- Growers should, where possible, retain a sub-sample of different seed batches for future testing purposes.

SCIENCE SECTION

Introduction

During early autumn 1998 concerns were raised by a number of growers regarding the occurrence of an apparent new disorder or disease of red beet. As crops neared maturity roots were observed to be severely distorted. In addition to the distortion, affected roots had an elongated neck and, in some cases, had a thickened tap root. One particular characteristic of the affected beet was a russetting or corkiness around the shoulder of affected plants. The smaller or 'baby beet' size grades were reported to be particularly badly affected. The syndrome was referred to as root malformation disorder or RMD. Various estimates have put the economic losses due to RMD at around £1M/annum in years when the problem has been particularly severe (1998 & 2002).

Following the initial occurrence of RMD HDC sponsored an investigation at Stockbridge House during 1999-2001 to try and determine the cause for the symptoms. Studies initially commenced on a broad basis to conduct a literature search, distribute a questionnaire to growers, conduct a series of pot studies and to eliminate a number of possible factors that could potentially have led to such severe root distortion. During this initial investigation, tests for 'Rhizomania' and other virus diseases were conducted, as were tests for herbicide injury, nematode infestation and bacterial pathogens. All tests proved negative. Subsequently, a series of replicated fungicide trials were undertaken, though whilst these provided some indication of a potential link with oomycete fungi, they were inconclusive due to the relatively low levels of RMD that occurred commercially during this period. However, on the basis of these initial findings recommendations were made to secure Off-Label approval for a series of oomycete fungicides via the HDC SOLA programme. As a result, a number of useful fungicides have subsequently gained approval for use on red beet and this at least improves the armoury of available fungicides on this relatively minor crop.

However, in October 2002, before such oomycete fungicides could be approved, growers, particularly in the Isle of Axholme region of South Yorkshire, again reported an extremely high incidence of RMD. On this occasion, it appeared that the problem developed quite late in the season (August-September). In some cases it was severe in fields that had not grown commercial crops in the Chenopodiaceae for several years or on land that had been down to grass for 20 years. As previously, the problem appeared to correlate closely with wet weather, in this case heavy rainfall during August after a prolonged dry spell. The reported absence of early symptoms and the presence of severe RMD in 'virgin' sites, tended to suggest aerial dissemination e.g. an air-borne, rather than a soil-borne, fungus. Close inspection of affected crops noted a fairly heavy infestation of downy mildew caused by *Peronospora farinosa* f. sp. *betae*, a pathogen not noted at particularly significant levels in previous years. As an oomycete fungus this obligate pathogen could also be expected to be well controlled (subject to the absence of resistant strains in the pathogen population) by SL567A. In other crops downy mildew fungi e.g. *Peronospora viciae* in peas are reported to infect seedlings systemically to cause distortion, without obvious sporulation. A web-based report from Oregon in the USA describes symptoms of d. mildew in red beet that correlates closely with those of RMD and this certainly required further investigation.

A further project initiated in 2003 investigated the role played by both soil- and air-borne pathogens in the RMD problem in a series of field-scale trials as a further means of elucidating the primary cause. The primary objective was to evaluate a soil sterilisation treatment in conjunction with a range of existing and novel fungicides. Separately, a search of past scientific literature on the subject was conducted. The aim here was to determine if there was any information available to ascertain whether the d. mildew pathogen found on

wild *Chenopodiaceae* possibly acted as a reservoir for subsequent infection of commercial 'beet', or indeed whether different host-specific pathovars were involved in the problem.

As a result of the mounting evidence of a possible association between infection by the d. mildew fungus (*P. farinosa*) and RMD symptoms discussions were opened with scientists at CSL. Following these initial discussions a separate project funded by HDC was instigated at CSL (Project Code M4EE 1000) during January-March 2004 to try to prove the hypothesis that the RMD affected roots were a result of a systemic invasion by the obligate oomycete pathogen *Peronospora farinosa*. This collaborative project with CSL was very successful and a valuable molecular (PCR) method for quantifying DNA of *P. farinosa* in red beet root tissues was developed. Furthermore, an initial validation test using RMD affected and unaffected red beet roots gave very positive results and this further strengthened the case for an association between RMD and d. mildew infection. The full results of this initial development work were reported in a previous report (FV 226c).

As a result of this positive development and to maintain the impetus further work was commissioned by HDC in Spring 2004, designed to evaluate a range of fungicides aimed at d. mildew control for their efficacy and timing in controlling RMD in red beet and also to fully integrate the novel PCR technique for quantifying the d.mildew fungus in distorted roots. The aim was to identify two high risk commercial sites to establish fungicide comparison trials (superimposed over untreated and Wakil treated crops) and fungicide timing trials (in the non-Wakil treated area of the crops). Separately, occasional commercial crops, subject to the development of either d. mildew and/or RMD were monitored, to further investigate any causal relationship using the molecular technique developed at CSL. The results of the fungicide timing and efficacy studies was somewhat inconclusive as no downy mildew was observed in any of the crops and very little RMD was detected during harvesting. The monitoring of other commercial crops proved to be extremely valuable and useful data was collected which demonstrated a strong correlation between foliar downy mildew infections, and the presence of DNA from *P. farinosa* in the distorted roots.

Throughout this period, investigations into RMD have continued to be hampered by a combination of factors, not least the unusual etiology of the disorder. This, in conjunction with the sporadic nature of the problem and the difficulty associated with obligate (non-culturable) pathogens such as downy mildew, have made elucidation of the RMD problem particularly challenging. However, due to the potential economic losses should the problem reappear and be uncontrolled commercially further funding was awarded in 2005. The objectives of the continuing investigation in 2005 were as follows:

1) **To undertake a single replicated fungicide trial in a commercial red beet crop in South Yorkshire to investigate the efficacy and timing of fungicides for effective control of RMD (STC)**

2) **To conduct further validation studies with the molecular assay through investigation of potential cross-reaction with oomycete and other potential pathogens of red beet to increase the confidence limits and further confirm the earlier detection of *Peronospora* in RMD affected roots (CSL)**

3) **To continue to respond to industry requests to monitor crops for d. mildew & RMD and to conduct *ad hoc* molecular assays on samples from STC and the industry, subject to the development of RMD and related symptoms in commercial crops during 2005 (STC & CSL)**

4) **To modify, validate and test the molecular (PCR) assay for the detection of *Peronospora* in commercial lots of red beet seed (STC & CSL)**

Materials & Methods

1. Replicated fungicide trial

Trial site location & trial design

The site chosen for the field experiment in 2005 was situated on a commercial red beet farm in the Isle of Axholme area of South Yorkshire in an area with a previous history of RMD. Red Beet cultivar Pablo was used throughout. The trial design comprised a randomised block with 6 replicates, split to include 3 replicates with Wakil treated seed and 3 replicates with Thiram treated seed (a trial plan is shown in Appendix 1).

Treatments

A tank mix of two experimental fungicides with known activity against downy mildew was applied to the trial area, with 'treatments' comprising different application timings (as in the timing trials in 2004). The fungicides used were Fubol Gold[1] (mancozeb + metalaxyl-M) and Invader[1] (dimethomorph + mancozeb).

Table 1. Timing of fungicide applications in 2005 field trial

Treatment	May		June		July		August		September	
	1*	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
1. Untreated control	-	-	-	-	-	-	-	-	-	-
2. Sprayed control	+	+	+	+	+	+	+	+	+	+
3. Two spray timing	+	+								
4. Two spray timing			+	+						
5. Two spray timing					+	+				
6. Two spray timing							+	+		
7. Two spray timing									+	+
8. Three spray timin	+	+	+							
9. Three spray timin				+	+	+				
10. Three spray timi							+	+	+	

** Optional timing depending on the drilling and harvest date of the selected crop. The first spray was actually applied in late May following drilling on 9th May. The crop was harvested slightly earlier than anticipated on 22nd September due to the absence of either downy mildew or RMD at the trial site.*

Spray application

Fungicide applications were carried out using a battery powered knapsack sprayer with a 4 nozzle boom. The sprayer was pressurised to a constant 2 bar pressure and F110/0.80/3 nozzles were used throughout.

Crop Diary

09.05.05 Trial area sown, cv. Pablo, sowing rate 1.5million/ha.
25.05.05 Trial area marked out, and 1st fungicide application made (T2, 3 & 8)
10.06.05 2nd fungicide application carried out (T2, 4 and 8)
23.06.05 3rd fungicide application carried out (T2, 4 & 9)
08.07.05 4th fungicide application carried out (T2, 5 & 9)

[1] These fungicide products were selected specifically for experimental use against oomycete fungi in the trial though are not currently approved for commercial use in red beet crops in the UK.

22.07.05 5th fungicide application carried out (T2, 5 & 9)
05.08.05 6th fungicide application carried out (T2, 6 and 10)
22.09.05 Half trial area (plots 1-30) harvested and assessed.

Disease assessments

Prior to each fungicide application a thorough visual assessment of the crop was carried out to check for the onset of any downy mildew or other infection on the foliage and/or roots.

At harvest, a 1m long strip from all 4 rows (1.83m wide) were harvested and assessed. The plants were assessed for the presence of visible downy mildew on the foliage, the number of distorted beet, and the severity of the distortion seen using the severity scale shown in Appendix 2.

2. Further validation studies with the molecular Taqman PCR assay

Previously the TaqMan assay had been designed to *P. farinosa* 28S ribosomal DNA sequence. One limitation of this assay is that the sequence available for *P. farinosa* is very limited. It was thought that this assay may cross react with other pathogens especially other oomycetes such as *Pythium*, *Phytophthora* and possibly *Aphanomyces* species. In order to further investigate the link between *P. farinosa* and root malformation disorder (RMD) it was necessary to rule out the possibility of misidentification by the TaqMan assay and also to eliminate the presence of any other fungal species that could induce RMD.

In recent years molecular characterisation and sequencing of fungal genes has greatly expanded. Using information gathered by other researchers, who have linked species designated by morphological characters to sequences of certain genes, it is possible to sequence these genes in an unknown sample and identify it to species level. The Internal Transcribed Spacer (ITS) Region and Cytochrome Oxidase I gene (COI) are commonly used for this purpose. With this approach it is possible to perform a PCR on a sample to amplify the DNA of a particular gene. The sequence of this DNA is then determined and compared to other sequences available on public access databases. The key to this approach is the use of PCR primers that will amplify DNA from a broad range of fungi. An adaptation of this approach has been used to try to identify any fungi that may be present in RMD affected beet.
Literature searches were performed to identify PCR primers that would amplify DNA from a wide range of fungi. A DNA sequence alignment was carried out for Beta tubulin genes from Oomycete sequences available on public access databases. This alignment was used to design a new PCR primer set to amplify beta tubulin DNA from Oomycetes. PCR primers previously designed to amplify the mitochondrial Cytochrome Oxidase I gene of fungi were tested against *Phytophthora* DNA from plate cultures to ensure they would amplify Oomycete DNA. Details of all PCR primers used are listed in Table 2.

Broad range PCR's were carried out with primers designed to the ITS region, 28S ribosomal DNA, Beta tubulin, COI and Cytochrome Oxidase II. These PCRs amplified the DNA of any fungi present including more than one species of fungi if present. Any amplified DNA would then be sequenced and compared to DNA sequence databases in order to identify any fungi present.

Table 2: PCR primers designed to amplify DNA from a broad range of fungi.

Target gene	Primer Name	Primer sequence 5′ to 3′	Source
Internal Transcribed Spacer Region	ITS1	TCCGTAGGTGAACCTGCGG	White *et al*. (1990)
	ITS4	TCCTCCGCTTATTGATATGC	
Beta Tubulin	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	Glass & Donaldson (1995)
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	
Beta Tubulin (Oomycete specific)	BtO F1	AGTGCGGTAACCARATTGGHGC	CSL
	BtO F2	AATGTGGGAATCARATCGGHGC	
	BtO R1	TTCCGTGAATTCCATRTCATCCATACC	
	BtO R2	CTCAGTGAACTCCATRTCGTCCATACC	
28S Ribosomal DNA	LSU-0025F	ACCCGCTGAACTTAAGCATAT	Petersen & Rosendahl (2000)
	LSU-1170R	GCTACTCTGAGGGAAATTTCGG	
Cytochrome oxidase I	ay347307 F	TTATTATATTTAWKKGATGAAGTWAT	CSL
	ay347307 R	GGTAWGCATCIRCTTTWATACCTAA	
Cytochrome Oxidase II	COII Oomycetes F	GGCAAATGGGTTTTCAAGATCC	Hudspeth *et al*. (2000)
	COII Oomycetes R	CCATGATTAATACCACAAATTTCACTAC	

PCR was performed on 20 DNA extracts from healthy beet roots, 20 from RMD affected beet roots, plus 8 extracts from 2004 testing year and 9 DNA extracts from red beet seed. Each of the 6 PCR primer sets was used to test each DNA extract.

3. Molecular assays on *ad hoc* samples from industry

Subject to the development of downy mildew and/or RMD symptoms in the trial crop the aim was to provide a supply of affected material for molecular testing to CSL to a. further validate the test itself and b. to increase the level of confidence that *Peronospora* is responsible for the RMD symptoms that have occurred previously in commercial beet crops. As it was, downy mildew and RMD both remained at low levels during 2005 only a small number of distorted roots and equivalent samples of non-distorted (healthy) roots were subsequently collected (including from the replicated trial area) and forwarded to CSL for analysis. Separately, in order to determine if *P. farinosa* can be detected on leaves growing from RMD affected roots, a small batch of distorted and non-distorted roots were grown-on at STC for further testing. Foliage produced from the affected and non-affected roots was harvested and forwarded to CSL for testing using the TaqMan assay to determine if this approach may prove more appropriate for future testing. A bulk sample was taken for analysis which included all leaves from each root. The samples were homogenised, sub-samples taken and DNA extracted. Each DNA extract was tested with the *P. farinosa* TaqMan assay.

4. Detection of *Peronospora* in commercial lots of red beet seed

In conjunction with the HDC Project Co-ordinator, efforts were made to secure a selection of various seed-lots from both the 2005 crop and previous crops for method development and molecular analysis at CSL. In total only 6 different seed-lots were sourced and submitted for testing as it was evident that growers did not retain seed from previous seasons and most

had drilled all the seed for the 2005 crop. From each seed-lot obtained, 10 DNA extractions were performed each containing 10 random seeds from each batch. These DNA extractions were tested with the modified *P. farinosa* TaqMan assay and the quantity of *P. farinosa* present in or on the seed determined.

Results

1. Replicated fungicide trial

No evidence of foliar downy mildew was observed in the trial area throughout the trial period. The grower confirmed that he had not seen any d. mildew in any of the red beet crops on his or surrounding farms during the season. In light of this information and the apparent absence of RMD in the root tissues spray applications were discontinued in mid-August as the beet were maturing (baby beet size), and it was considered that any subsequent infection with d. mildew after this date was unlikely to influence the level of root distortion. A harvest assessment was undertaken (plots 1-30) on the 22nd September 2005 to ascertain the level of distortion within the trial area.

Table 3. Harvest data from plots 1-30 collected on 22nd September

Treatment	No. of plants with visible d. mildew	Mean no. of distorted roots/plot	Mean severity of distortion of affected roots (0-5 scale)	Mean harvest weight (tonnes/ha)
1. Untreated control	0	0.3	4	61.2
2. Sprayed control	0	0	0	57.5
3. Two spray timing 1	0	0	0	65.8
4. Two spray timing 2	0	0	0	65.5
5. Two spray timing 3	0	0.3	3	63.1
6. Two spray timing 4	0	0	0	65.1
7. Two spray timing 5	0	1.0	4	54.4
8. Three spray timing 1	0	0	0	62.4
9. Three spray timing 2	0	0.3	5	59.2
10. Three spray timing	0	0.3	3	65.8

No d. mildew sporulation was observed on the plants harvested. A total of 7 distorted roots were recorded across the 30 plots, representing approximately 0.2% infection across the trial area. None of the various application timings appeared to have had any impact on the yields, with all treatments providing similar weights.

2. Further validation studies with the molecular Taqman PCR assay

Using an *ad hoc* sample of distorted red beet roots collected randomly from a commercial crop at harvest which showed a low incidence of the disorder 20 RMD affected and 20 healthy roots were analysed. DNA extractions were performed on each root individually; these extractions were tested with the *P. farinosa* TaqMan assay. As expected from previous studies, very little *P. farinosa* was found in the healthy roots with only one root positive with a very small quantity of *P. farinosa*. Of the affected beets 16 were positive by the *P. farinosa* assay with quantities ranging from 0.0003 to 1.65, with the average amount found to be 0.43. One affected red beet was negative; three DNA extracts from affected beets failed and contained either no DNA or contaminating inhibitors preventing the PCR reaction from taking place (Table 4).

Table 4: Results of testing healthy and RMD affected red beet roots* with the *P. farinosa* TaqMan assay.

Sample name	Average *P. farinosa* Ct value	Relative Quantity of *P. farinosa*	Sample name	Average *P. farinosa* Ct value	Relative Quantity of *P. farinosa*
Healthy 1	-	0	Affected 1	29.76	0.26
Healthy 2	-	0	Affected 2	30.47	0.32
Healthy 3	-	0	Affected 3	30.07	0.22
Healthy 4	39.73	0.0005	Affected 4	30.49	0.097
Healthy 5	-	0	Affected 5	30.33	0.074
Healthy 6	-	0	Affected 6	39.43	0.00033
Healthy 7	-	0	Affected 7	33.21	0.034
Healthy 8	-	0	Affected 8	30.24	0.25
Healthy 9	-	0	Affected 9	29.34	0.61
Healthy 10	-	0	Affected 10	29.81	0.29
Healthy 11	-	0	Affected 11	30.91	1.16
Healthy 12	-	0	Affected 12	31.42	0.16
Healthy 13	-	0	Affected 13	-	-
Healthy 14	-	0	Affected 14	32.66	0.18
Healthy 15	-	0	Affected 15	29.73	1.08
Healthy 16	-	0	Affected 16	-	-
Healthy 17	-	0	Affected 17	-	0
Healthy 18	-	0	Affected 18	-	-
Healthy 19	-	0	Affected 19	34.40	0.45
Healthy 20	-	0	Affected 20	29.42	1.65

* Samples tested were from an *ad hoc* sample collected during the harvesting of a commercial crop which exprtesed a low incidence of RMD.
Note. The Ct value is the cycle at which the fluorescence in the reaction, and therefore build up of amplified DNA, crosses a pre-set threshold level. The Ct value is directly related to quantity of *P. farinosa.*

PCR using ITS primers

A PCR product (approximately700bp in length) was amplified from 46 out of the 57 extracts tested; although there was no pattern to relate healthy vs. affected beet with the presence or absence of a band. It was suspected that the resulting product corresponds to the ITS region of the red beet itself rather than any fungal gene present in the sample. To confirm this 4 PCR products from affected beet and 4 from healthy beet were cloned into a plasmid vector, the inserts from several randomly selected clones were sequenced and the identity of the sequence confirmed by BLAST searches and DNA alignments with known genes. All ITS sequences isolated from either affected or healthy red beet were identified as originating from red beet. This PCR did not identify any fungi present in the sequence.

PCR using 28S Ribosomal DNA primers

The same extracts were used in PCR with primers designed to 28S ribosomal DNA a band was produced from 48 of the 57 extracts. Again no pattern was seen between affected vs. healthy red beet and the presence or absence of a DNA band. The bands were all the same size at approximately 950bp. However this gene in oomycetes is approximately 1165bp. The DNA amplified in this reaction is assumed by its size to be the corresponding plant gene and no further action was taken.

PCR using other primer sets

When PCR was carried out using primers designed to the COI gene and Oomycete Beta Tubulin gene no DNA was amplified. COII primers and standard Beta Tubulin primers

produced spurious non-specific amplification with no correlation between affected vs. healthy red beet and the presence or absence of a DNA band.

The use of broad range PCR did not detect any additional fungi present in RMD affected red beet samples, even though the TaqMan assay shows that *P. farinosa* was present in the affected beet. TaqMan assays are by their nature more sensitive than conventional PCR assays; the small size of TaqMan amplicons allows for very efficient amplification whereas conventional PCR assays produce larger amplicons. This means that TaqMan can often detect the presence of a DNA sequence where traditional gel based PCR has failed to detect it.

The 28S PCR primers were previously used in 2004 to confirm the presence of *P. farinosa* in affected red beet. It was found during this testing that DNA extracts from affected red beets with a TaqMan Ct value greater than 27 would give no product by conventional gel based PCR. Although *P. farinosa* is present in the sample conventional PCR would not be sensitive enough to detect it. The Ct values obtained from affected red beet samples from the 2005 season, used in this study, were all greater than 29 (table 5) and therefore it is unlikely that any fungi detected in these samples by the TaqMan assay would be detected by the 28S PCR. In essence the amount of fungi present is too low to be detected by 28S PCR although within the detection limits of TaqMan.

PCR using primers very specific to fungal pathogens, COI, COII and Beta tubulin did not amplify fungal DNA. ITS and 28S PCR primers only amplified corresponding genes from the red beet genome. No additional fungal pathogens were detected by broad range PCR. Further in-silico analysis of the DNA sequence amplified by the TaqMan assay shows that *Aphanomyces cochlioides* a fungus known to affect beet is very unlikely to be detected by the TaqMan assay. No fungi could be detected in RMD affected samples other than by the *P. farinosa* TaqMan assay, further strengthening the hypothesis that the fungi detected by TaqMan is that which causes RMD.

3. Molecular assays on *ad hoc* samples from industry

Due to the very low incidence of both d. mildew and RMD in the 2005 season very few *ad hoc* red beet samples were sent to CSL for TaqMan testing during 2005. Samples that were submitted were collected from the fungicide efficacy/timing trial carried out on a commercial farm and these comprised a small no. of distorted roots together with an equivalent sample of visibly healthy roots from the same trial. No *ad hoc* samples from other growers were collected or received and this further reflects the very low level of observed d. mildew and RMD seen during the season.

As in previous years, all results of TaqMan testing are presented as relative quantities of *P. farinosa* compared to a positive control comprising RMD affected beet roots (recorded as 1.0). In the results tabulated below all figures with a value above 0 are positive for *Peronspora* DNA; only values of 0 are negative and contain no *Peronospora* DNA.

The results of the molecular tests on the trial samples is shown in Table 5 below.

Table 5*:* Results of TaqMan testing of red beet samples sent to CSL.

Sample name	Relative Quantity of *P. farinosa*
Red beet 1	0.079

Red Beet 9	0.25
Red Beet 10	0
Red beet 13	4.45
Red Beet 24	0.013
Red Beet 26A	0
Red Beet 26B	0.0045
Healthy	0

The TaqMan assay was also used to determine whether it was possible to detect d.mildew DNA in samples of foliage grown on from distorted beet. In this experiment very low quantities of *P. farinosa* were detected from one healthy plant (quantity =0.00037) and three plants where the foliage came from RMD affected plants (quantities ranging from 0.00013 to 0.0013) (Table 6).

Table 6: Testing of leaves from healthy and RMD affected roots using the *P. farinosa* TaqMan assay

Sample	Relative Quantity of *P. farinosa*	Sample	Relative Quantity of *P. farinosa*
Healthy leaves 1	0	Affected leaves 1	0.0013
Healthy leaves 2	0	Affected leaves 2	0
Healthy leaves 3	-	Affected leaves 3	0
Healthy leaves 4	0	Affected leaves 4	0.00030
Healthy leaves 5	0	Affected leaves 5	0.00013
Healthy leaves 6	-	Affected leaves 6	0
Healthy leaves 7	0	Affected leaves 7	-
Healthy leaves 8	0.00037	Affected leaves 8	0
Healthy leaves 9	-	Affected leaves 9	-
		Affected leaves 10	0

From this data it would seem unlikely that significant detection of *P. farinosa* could be achieved by sampling leaves although the sample size of this experiment was very small.

4. Detection of *Peronospora* in commercial lots of red beet seed

Of the 60 extracts (6 x 10 sub-samples) from red beet seed-lots only 3 (5%) were truly negative whilst *P. farinosa* was present in 57 (95%) of the samples. The quantities of *P. farinosa* detected were highly variable both between and within the 6 seed-lots. In general, the quantity of *P. farinosa* was lowest in Pablo F1 (Lot. No. E01667) and highest in "Old seed". It is however worth noting that a positive result by the TaqMan assay does not necessarily indicate presence of a viable pathogen as the TaqMan assay will also detect DNA from dead cells. A summary of the seed-test results is shown in Table 4. (Full results in Appendix 3)

Table 4: TaqMan testing of red beet seed-lots for the detection of *P. farinosa.*

Description of sample	Average quantity *P. farinosa* determined by

	TaqMan
Variety:Detroit 2 ELS Crimson Globe. Lot. No. E02026. Treatments: Thiram soak, Wakil XL-Cymoxanil, Metalaxyl-M, Fludioxonil	1.12 ± 2.39
Variety:Pablo F1. Lot. No. E01667. Treatments: Thiram soak, Wakil, XL-Cymoxenil, Metalaxyl-M, Fludioxonil	0.36 ± 0.52
Variety:Darko. Treatments: Thiram soak, Cymoxanil, Fludioxonil, Metalaxyl-M.	7.33 ± 18.22
Variety:Pablo F1. Lot No. E01050 Treatments: Thiram soak. Early Drilling +1/2 trial plot	2.27 ± 4.24
Variety:Detroit 2 ELS Crimson Globe. Lot. No. E02303. Treatments: Thiram soak, Wakil, XL-Cymoxanil, Metalaxyl-M, Fludioxonil	7.67 ± 24.12
Old seed Back of Russ Meadlands + stout 125	19.95 ± 46.97

It is important to recognise that the results for the *Peronospora* seed testing reported here are provisional and do not necessarily signify a seed-borne cause for RMD. However, these initial studies do require following up in more detail. It is highly pertinent here that the Taqman assay will detect DNA of a specific pathogen in both living and dead cells so, even if these preliminary results were confirmed, we could not necessarily conclude that it would automatically lead to a systemic infection by the downy mildew pathogen (or RMD symptoms at harvest).

Conclusions

- Downy mildew (*P. farinosa* f.sp. *betae*) was generally present at low to negligible levels in commercial crops during 2005.

- Root distortion (RMD) levels were very low commercially in 2005 and reports from growers suggest that typically less than 1-2% of graded roots were affected. Indirectly this supports the hypothesis of a possible link between early systemic infection by *P. farinosa* and RMD development on the roots later in the season.

- A fully replicated fungicide timing trial was carried out at Westwoodside in 2005. No evidence of d. mildew infecting the untreated plots in the trial area was observed. Following harvest only negligible levels of root distortion were found comprising a total of 7 affected roots. Following molecular testing 5 out of the 7 distorted roots were found to contain *P. farinosa* DNA. No DNA was detected in an equivalent non-distorted root sample from the same crop.

- No evidence of any phytotoxicity effects were seen on plants at the trial site following application of the various experimental fungicide treatments.

- DNA of *P. farinosa* was detected in variable amounts on 6 batches of seed using the TaqMan assay. However, as the test cannot distinguish between DNA from dead or viable cells this information must be treated with caution. Also, there remains a slight possibility of cross- reaction with some other fungus on or in the seed and therefore the results must be regarded as preliminary at this stage.

- A broad range of other PCR techniques were evaluated by CSL to investigate whether fungal DNA from other oomycete fungi (e.g. *Phytophthora* spp., *Pythium* spp. and *Aphanomyces* spp.) was present in red beet root or seed samples. The tests did not show the presence of any fungal DNA. These tests were also negative for *P. farinosa* and this suggests perhaps that these other tests are less sensitive and therefore of limited value in finally confirming *Peronospora* as the primary incitant of RMD in red beet.

- Separate work here also provided further evidence to support the view that it is unlikely that the detection of *P. farinosa* DNA in distorted roots is due to a cross-reaction with *Aphanomyces cochlioides*. However, whether such cross-reactions between other oomycete fungi such as *Pythium* or *Phytophthora* spp. occur this has not been conclusively proved in these studies and this continues to hamper interpretation of the data.

Technology Transfer

As in previous years the information from this project has been relayed to the industry throughout the season via the Red Beet Technology Group meetings, in one-to-one contact with growers and the various activities of both the HDC Project Co-ordinator and the project team.

In addition, various articles have been published in HDC News and the trade press to update the industry of progress. An HDC Fact sheet is currently in preparation.

References

Byford, W J (1967). Host specialisation of *Peronospora farinosa* on *Beta*, *Spinacia* and *Chenopodium*. *Transactions of the British Mycological Society* **50** (4), 603-607.

Cook, H T (1936).Cross-inoculation and morphological studies on the *Peronospora* species occurring on *Chenopodium album* and *Spinacia oleracea*. *Phytopathology* **26**, 89-90.

Danielson, S (2001). Heterothallism in *Peronospora farinosa* f. sp. *chenopodii*, the causal agent of downy mildew of quinoa (*Chenopodium quinoa*). *Journal of Basic Microbiology* **41** (5), 305-308.

Danielson, S; Bonifacio, A & T Ames (2003). Diseases of quinoa (*Chenopodium quinoa*). *Food Reviews International* **19** (1-2), 43-59.

Dzhanuzakov, A (1962). Specialisation and variability in some Peronosporaceous fungi. *Bot.Zh. SSSR* **47**, 862-866. (In *Review of Applied Mycology* **42**, 4 (1963)).

Frinking, H D; Harrewijn, J L & C F Geerds (1985). Factors governing oospore production by *Peronospora farinosa* f sp *spinaciae* in cotyledons of spinach. *Netherlands Journal of Plant Pathology* **91** (5), 215-223.

Frinking, H D & E G A Landers (1986).A comparison of two pathosystems; downy mildew on *Spinacia oleracea* and on *Chenopodium album*. *Netherlands Journal of Plant Pathology* **92** (3), 97-106.

Fuckel, L (1865). *Peronospora schactii* n.sp. *Fung. Rhen*. 1508.

Glass and Donaldson (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology*, **61** (4), 1323-1330.

Hiura, M (1929). Studies on some downy mildews of agricultural plants III. On the downy mildew of spinach. *Agriculture Hort.., Tokyo* **4**, 1394-1406.

Hudspeth, Nadler and Hudspeth (2000). A COX2 molecular phylogeny of the peronosporomycetes. *Mycologia* **92** (4), 674-684.

Irish, B M; Correll, J C; Koike, S T; Schafer, J & T E Morelock (2003). Identification and cultivar reaction to three new races of the spinach downy mildew pathogen from the United States and Europe. *Plant Disease* **87** (5), 567-572.

Leach, L D (1931). Downy mildew of the beet caused by *Peronospora schactii* Fuckel. *Hilgardia* **6**, 203-251.

Petersen AB, Rosendahl S (2000). Phylogeny of the Peronsporomycetes (Oomycota) based on partial sequences of the large ribosomal subunit (LSU rDNA). *Mycological Researc*h, **104** (11), 1295-1303.

Spencer, D M (1981). The downy mildews. Academic Press, London. 636 pp.

Vanasch M A J & H D Frinking (1988). Heterothallism in *Peronospora farinosa* f. sp *spinaciae*. *Transactions of the British Mycological Society* **91**, 692-693.

Weller SA, Elphinstone JG, Smith NC, Boonham N, Stead DE (2000). Detection of Ralstonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Applied and Environmental Microbiology*, **66** (7), 2853-2858 JUL.

White, Bruns, Lee and Taylor (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols a guide to methods and applications. Eds Innis, Gelfand, Sninsky, White. Academic Press Inc., New York, p315-322.

Wright, C M & W D Yerkes (1950). Observations on the overwintering of the pathogen causing downy mildew of spinach in the Wala-Walla area. *Plant Disease Reporter* **34**, 28.

Yerkes, W D & C G Shaw (1959). Taxonomy of the *Peronospora* species on *Cruciferae* and *Chenopodiaceae*. *Phytopathology* **49**, 499-507.

Acknowledgements

Our thanks and grateful appreciation goes to Graham Smith at South Carr Farm for his continuing enthusiasm, help, support and advice during the field phase of this trial. We are also grateful to Neil Boonham and Kathy Walsh at the Central Science Laboratory, York, for carrying out the molecular analyses on the samples of red beet and seed throughout the season. Finally, many thanks to the many growers, consultants and trade representatives who have helped in one way or another to aid resolution to the on-going problem of RMD in the red beet crop.

Appendices

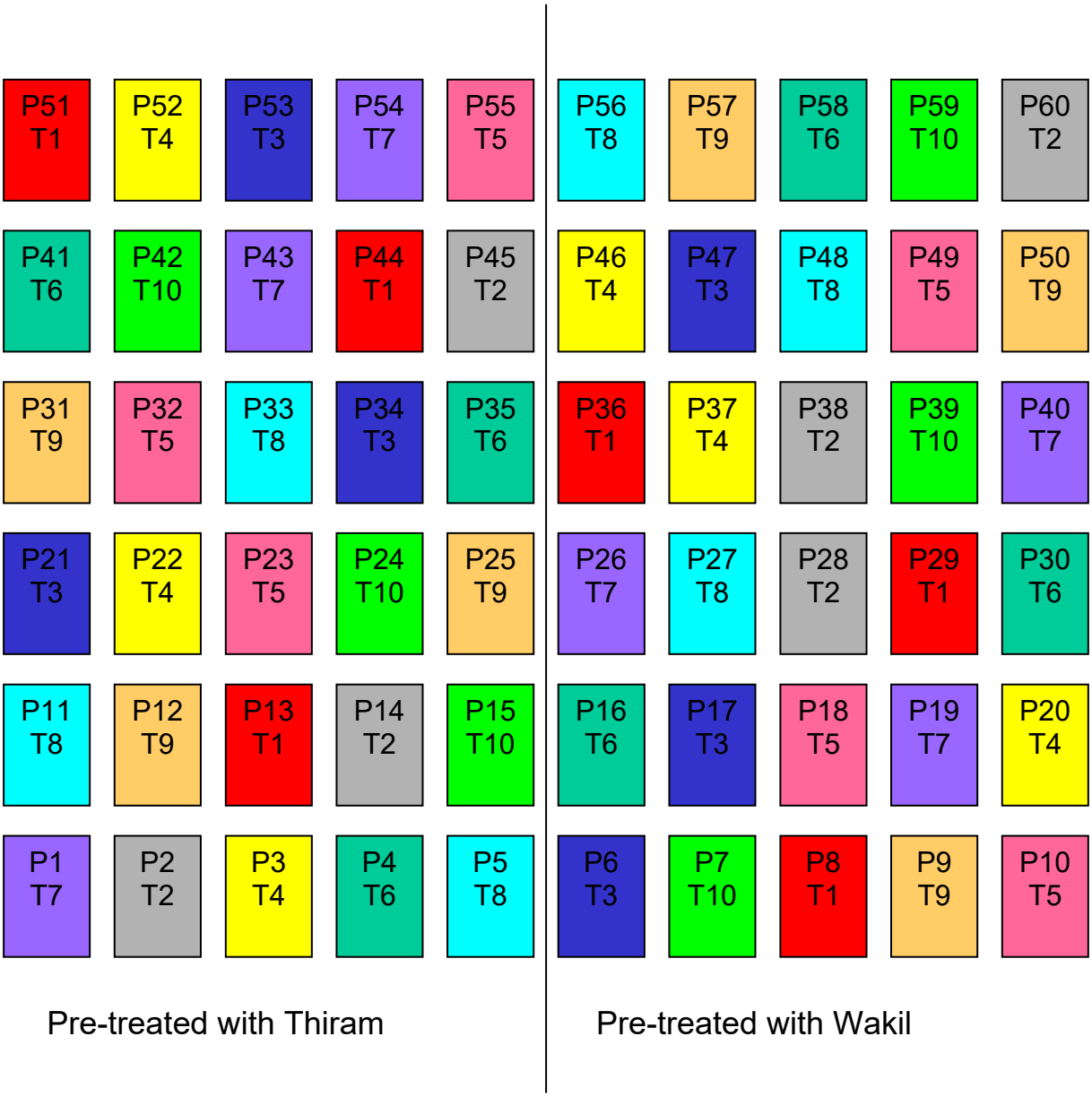
Appendix 1 : Fungicide Timing trial plan
Appendix 2 : Assessment scale for RMD symptoms
Appendix 3 : Full data for seed testing using TaqMan *P. farinosa* assay.

Appendix 1: Fungicide timing trial plan

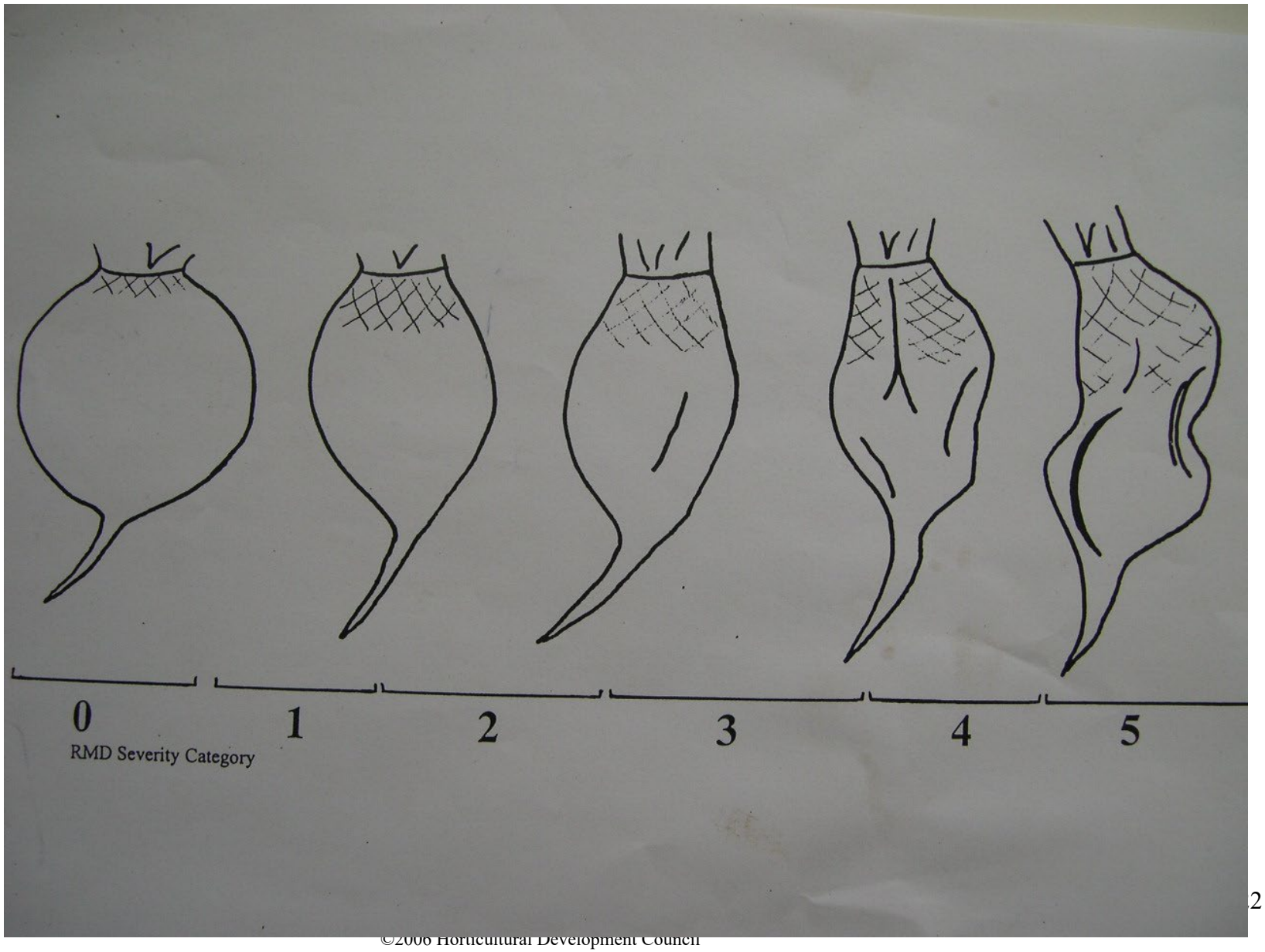
Red Beet– Fungicide Timing Trial 2005
Cv. Pablo Carr Farm - Westwoodside

Treatments

Fubol Gold: 1.9kg/ha
Invader: 2kg/ha
Tank mix: 250lt/ha water



Appendix 2- Assessment scale for RMD symptoms



Appendix 1: Full data of seed testing using TaqMan *P. farinosa* assay.

Description of sample	Extract name	Quantity of *P. farinosa* relative to positive control	Average quantity *P. farinosa* per seed-lot	Standard deviation
Variety:Detroit 2 ELS Crimson Globe. Lot. No. E02026. Treatments: Thiram soak, Wakil XL-Cymoxanil, Metalaxyl-M, Fludioxonil	A1	7.740	1.12	2.39
	A2	0.658		
	A3	0.046		
	A4	0.001		
	A5	1.333		
	A6	0.045		
	A7	0		
	A8	0		
	A9	0.003		
	A10	1.327		
Variety:Pablo F1. Lot. No. E01667. Treatments: Thiram soak, Wakil, XL-Cymoxenil, Metalaxyl-M, Fludioxonil	B1	0.037	0.36	0.52
	B2	0.049		
	B3	No DNA		
	B4	0.910		
	B5	0.017		
	B6	1.420		
	B7	0.018		
	B8	0.027		
	B9	0.062		
	B10	0.715		
Variety:Darko. Treatments: Thiram soak, Cymoxanil, Fludioxonil, Metalaxyl-M.	C1	11.687	7.33	18.22
	C2	0.183		
	C3	1.112		
	C4	0.674		
	C5	0.272		
	C6	0.521		
	C7	0.279		
	C8	0.011		
	C9	0.383		
	C10	58.203		
Variety:Pablo F1. Lot No. E01050 Treatments: Thiram soak. Early Drilling +1/2 trial plot	D1	0.012	2.27	4.24
	D2	10.558		
	D3	0.377		
	D4	0.255		
	D5	0.035		
	D6	Little DNA		
	D7	0.068		
	D8	0.122		
	D9	0.170		
	D10	8.863		
Variety:Detroit 2 ELS Crimson Globe. Lot. No. E02303.	E1	0.042	7.67	24.12
	E2	0.058		
	E3	0.026		

Treatments: Thiram soak, Wakil, XL-Cymoxanil, Metalaxyl-M, Fludioxonil	E4	0.069		
	E5	0.012		
	E6	0.028		
	E7	0.048		
	E8	0.034		
	E9	76.333		
	E10	0.075		
Old seed Back of Russ Meadlands + stout 125	F1	0.008	19.96	46.97
	F2	9.680		
	F3	5.603		
	F4	0.010		
	F5	0.009		
	F6	10.910		
	F7	152.655		
	F8	16.581		
	F9	4.093		
	F10	0		