Final Report

Effect of Contrasting Irrigation Regimes on Populations of *Streptomyces* and Potential Antagonists and Control of Common Scab

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1. SUMMARY

A previous Defra LINK project, with industry support from PCL, had developed diagnostic tools for monitoring *Streptomyces* populations on potato tubers under field conditions based on detection of specific DNA sequences. These methods have been used to measure levels of pathogenic *Streptomyces* spp., the causal agents of common scab of potato, as well as total levels of actinomycetes as an indicator of total soil biotic activity. Thus it has been possible to quantify the level of suppression afforded by irrigation, allowing recommendations to be made for more precise and economical application of irrigation water to control scab. Our aims in this project were further examination of *Streptomyces* populations at time points within the window for scab control by irrigation defined under the previous project, and collect information on the level of scab at in tubers of different varieties under contrasting irrigation regimes. We also aimed to use samples taken from these trials as well as from experiments conducted under the previous LINK project to identify populations of potentially antagonistic microorganisms present in soils where scab levels were lower.

Field experiments were conducted at CUF in 2009, using the varieties Maris Piper, Hermes and Vales Sovereign. Five irrigation regimes were used, either irrigation at 20 mm SMD throughout the experiment, 20 mm SMD throughout except for between four and six weeks after tuber initiation, over-irrigation for three weeks post tuber initiation, over-irrigation between 10 and 13 weeks post initiation, and unirrigated. Tubers were sampled weekly from one to five weeks after initiation to determine *Streptomyces* populations, and at harvest to measure yield and scab level. Samples from this and previous trials were also used in DNA sequencing experiments to identify components of the microbial communities which may have potential to inhibit the onset of scab.

Control of scab was most effective when plots were maintained at field capacity following tuber initiation, although over-irrigation was detrimental to canopy duration and yield. In dry soils, populations of pathogenic *Streptomyces*, the causal agents of common scab, increased between three and four weeks after tuber initiation. Levels were lower in irrigated soils. The critical period for control of scab by irrigation was between one and three weeks following tuber initiation, although further investigation is needed before this can become the basis of control recommendations.

DNA sequencing was used to identify groups of microorganisms which may be responsible for suppression of scab where low populations of pathogenic *Streptomyces* (and hence low levels of scab) have been observed. Although no single group of microorganisms was shown to be prevalent at all of the diverse sites studied, some groups of bacteria were found to be associated with suppression of scab in more than one trial. Further research is now needed to determine how these potential biological control agents interact with *Streptomyces*.

2. INTRODUCTION

Work on common scab in the previous three seasons as part of the Defra-LINK programme showed that our knowledge of the mechanism of control of populations of pathogenic *Streptomyces* species under contrasting soil water regimes is rudimentary and further work was needed to look at possible antagonists to *Streptomyces*. A grant was obtained from the Potato Council for Fera and CUF to investigate the antagonist theory in greater detail. Greenvale AP were interested in testing their new variety Vales Sovereign alongside established varieties, particularly in relation to optimum common scab control irrigation regimes and water use efficiency. An experiment was conducted at CUF in 2009 to examine all of these factors in three varieties.

We have shown that levels of scab can be correlated with populations of pathogenic *Streptomyces* as well as total Actinomycetes, and that control by irrigation influences these populations to suppress disease. It is likely that this suppression is mediated by the wider soil microflora, and that populations of soil microbes, as yet unidentified, respond to irrigation and exert a suppressive effect on pathogenic *Streptomyces*. Initial experiments towards the end of a previous Defra LINK project, co-funded by Potato Council, used newly-developed DNA sequencing methods to identify specific populations of soil-inhabiting bacteria that respond to irrigation and may be mediating the suppressive effect of irrigation. Such is the novelty of these methods that the DNA sequencing studies were only available for use at the end of the project, and our aim in this project was to widen the use of these methods to examine changes in soil microflora in a larger set of field extracts. The aim of our approach is to identify microbial taxa associated with suppression of bacterial scab, which may constitute a causal link between irrigation practices and control of scab, and ultimately to use this information to design agricultural practices which enhance populations of suppressive organisms. We wished to determine whether irrigation-induced changes in microflora, which may be responsible for pathogen suppression, shared common features in different environments, or whether responses to irrigation were more diverse.

Pyrosequencing is a powerful method capable of generating many thousands of DNA sequences from a single sample. We used amplicon pyrosequencing, in which a known DNA fragment is amplified from all members of a population, and all DNA fragments represented in the sample are sequenced in parallel. This methodology affords a detailed examination of microbes present in environmental samples at a given time point.

3. MATERIALS AND METHODS

3.1. Irrigation Trials

The experiment was a fully-randomized factorial design, with all combinations of five irrigation regimes (rainfed only, I-; irrigated at 20 mm SMD throughout, I; unirrigated for 2 weeks between 4 and 6 weeks post-tuber initiation otherwise as I, I- 4-6; over-irrigated from tuber initiation for 3 weeks otherwise as I, I+ 0-3; over-irrigated between 10 and 13 weeks post-tuber initiation otherwise as I, I+ 10-13) and three varieties (Hermes; Maris Piper; Vales Sovereign). There were three replicate blocks.

The experiment was planted on 14 April 2009 using 35-40 mm M Piper SE 1, 35-40 mm V Sovereign SE2 and 40-45 mm Hermes E1 seed at a within-row spacing of 25 cm and 12 cm deep into pre-formed ridges, which were raked after planting to re-form the original ridge. Plots were 8 m in length and eight rows (6.10 m) or four rows (I+ 10-13) wide. A concentrated (34.6 % vol.) solution of ammonium nitrate was applied at a rate of 180 kg N/ha post-planting.

Irrigation was scheduled using the CUF Potato Irrigation Scheduling Model based on meteorological data obtained from a Delta-T Devices weather station 200 m from the experiment. The irrigation was timed based on the mean soil moisture deficits in the I treatments of M Piper and V Sovereign as there was little difference in emergence between these two varieties. Overhead irrigation was applied through a boom (RST Irrigation) and hose reel (Perrot SA, SH63/280) combination. Plots were differentially irrigated by turning nozzles on or off along the length of the boom. Nozzles were spaced at *c*. 0.5 m, so individual plots could be irrigated. Mean irrigation amounts were estimated from 24 raingauge readings per irrigation treatment, situated at ground level and not shielded by foliage.

Sprinklers (Dan Modular Small Swivel Yellow Anti-mist nozzles) were used to provide additional irrigation for I+ 0-3 and I+ 10-13 treatments. Sprinklers on 1 m risers were installed in every alternate furrow at 1 m spacing to form a grid pattern in the plot. They were adjusted to run at very low pressure (*c*. 0.5-0.6 bar) to reduce the risk of misting and drift into adjacent plots. The sprinkler systems were calibrated at the beginning of the season to determine flow rates at various pressures between 0.4 and 0.6 bar and charts created relating application rates in mm/hour to inlet pressure and number of plots being irrigated simultaneously. Irrigation for over-irrigated plots was twice daily (07:00 and 19:00 h) with a total of 5 mm/day, to ensure that plots were kept at, or above, field capacity. Irrigation amounts applied are detailed in Table 3.1 (below). The timings of the over-watered periods were based on the exact dates of tuber initiation for each variety rather than a mean of all varieties.

	Irrigation regime				
Date	I-	I	I- 4-6	I+ 0-3	I+ 10-13
Rainfall	227.0	227.0	227.0	227.0	227.0
2 June		21.1	21.1	21.1	21.1
2-22 June				105.0	
19 June		21.2	21.2	21.2	21.2
25 June		23.2	23.2	23.2	23.2
3 July		24.2		24.2	24.2
13 July		24.2		24.2	24.2
10-30 August					105.0
17 August		24.2	24.2	24.2	24.2
25 August		24.2	24.2	24.2	24.2
Total	227.0	389.3	340.9	494.3	494.3

TABLE 3.1. TOTAL RAINFALL AND IRRIGATION (MM) APPLIED DURING THE SEASON FROM EMERGENCE TO FINAL HARVEST

In each plot of one replicate block, soil water content was measured at 15 minute intervals using a Delta-T Devices Theta Probe ML2 permanently installed at emergence in the centre of the ridge mid-way between two adjacent plants at a depth of 15 cm and logged using a Delta-T Devices DL2 logger.

Plant emergence was recorded every 1-2 days in each plot by counting the number of plants emerged in two harvest rows. Tuber initiation was determined by digging two plants per plot every 2 days from 10 days after 50 % plant emergence and recording a plant as having initiated tubers if one or more stolons had swollen to twice their diameter at the tip. Ground cover was measured weekly after emergence until final harvest using a grid in two positions in each plot.

Samples of 10 tubers from 5 plants per plot were sent to Fera, York in perforated plastic bags 1, 2, 3, 4 and 5 weeks after tuber initiation for determination of *Streptomyces* populations. Tubers were sampled from I-, I and I+ 0-3 and M Piper and V Sovereign plots only. Populations of total Actinomycetes and pathogenic *Streptomyces* were assessed by quantitative real-time PCR using assays for 16S rDNA and the *txt* pathogenicity gene (which is common to pathogenic *Streptomyces*) respectively.

A final harvest of 12 plants was taken on 9 September and the tubers assessed for incidence and severity of common scab in the categories: 0, 0-1, 2-5, 6-25, 26-60 and 61-100 % surface area infected with scab.

Variates were analysed by analysis of variance using the GenStat Release 6.1 statistical package (Payne *et al*. 2002). Treatment means are stated to be significantly different only if the probability of differences occurring by chance were less than 5 % ($P < 0.05$). All error bars in figures are one standard error (S.E.) in length. The respective degrees of freedom (D.F.) are given in tables or figures where standard errors (S.E.) are presented.

Owing to the large populations of *Streptomyces* observed on the surface of tubers, the raw data relating to copies of DNA/g of peel were log-converted before conducting analysis of variance. One of the major hypotheses of control of common scab by irrigation is that in wet soils the ratio of pathogenic to non-pathogenic *Streptomyces* is decreased compared with dry soils, whether as a consequence of resource competition or antibiotic production by non-pathogenic *Streptomyces* is unclear

(Adams & Lapwood 1978; Neeno-Eckwall *et al.* 2001). Since there were some plots where apparently no *txtA* DNA was observed, the ratio could not be calculated. A better approach was suggested by Rodger White of the Statistics Group at the Centre for Mathematics and Computational Biology, Rothamsted Research (personal communication) which involved analysing the population data for both strains together using a split-plot analysis. The main assumption is that the extracted DNA is assayed separately for 16S and *txtA*. At the split-plot level, differences on the log-transformed data are equivalent to ratios but it is also possible get an indication of whether or not there are interactions at the split-plot level for 16S and *txtA*. This is better than analysing the ratio of *txtA* : 16S directly. In order to deal with zero values, the data had 1 added to the population prior to log-transformation. In figures presenting data on *Streptomyces* populations, it is difficult to represent standard errors (S.E.) in a meaningful way if y-axis scales are logarithmic. Therefore, the text explains whether there were significant differences in populations at a particular sampling and the error bars have been positioned in figures so that they give an indication of the error variation between treatments.

3.2. DNA sequencing methods for identification and quantification of potential antagonists

The project aimed to conduct a quantitative analysis of soil microflora potentially antagonistic to scab-causing *Streptomyces* spp using a pyrosequencing approach. Primers were designed to amplify the V1 and V2 variable regions of bacterial 16S and used to create a library of amplicons from *c.*90 trial plots, including the 2009 trials at CUF described here, trials at CUF in 2008 and plots on commercial sites (provided by Branston and QV foods) sampled during the previous Defra LINK project. These 16S libraries were sequenced at the next-generation sequencing facility at Fera.

DNA extracts made from tuber peel samples for determination of 16S and *txt* levels were used as templates to amplify conserved microbial genes. For bacteria and archaea these were variable regions of the 16S rDNA, which encodes the conserved ribosomal structural RNAs, and for fungi the internally transcribed spacer (ITS) region. PCR primers were designed which incorporated adaptor sequences for 454 sequencing. Amplicons were prepared from DNAs from field trials at CUF in 2008 and 2009, as well as trials conducted on commercial fields (at Branston and QV Foods field sites) during the 2008 growing season. Amplicons were sequenced in parallel using a Roche GS FLX DNA sequencer at Fera.

4. RESULTS

4.1. Emergence and tuber initiation

There was a small difference in the date of 50% plant emergence for M Piper (21 May) and V Sovereign (23 May) but there was a protracted period of emergence over the experimental area so that initial emergence (13 May) to 95 % emergence took 20 days. Hermes was much later in commencing emergence than the other two varieties, reaching 50 % emergence on 31 May, with initial emergence (22 May) to 95 % emergence taking 22 days. This protracted emergence had a consequential effect on tuber initiation. Maris Piper commenced initiation on 31 May, 18 days after initial emergence. The date of 50 % initiation was 4 June, 15 days after 50 % emergence. All sampled plants had initiated by 8 June. In V Sovereign, initiation commenced on 2 June (20 days after initial emergence), with 50 % of plants initiated on 6 June (14 days after 50 % emergence). All sampled plants had tubers by 10 June. Hermes commenced initiation on 9 June (18 days after initial emergence) and reached 50 % initiation on 13 June (13 days after 50 % emergence). On 17 June, all sampled plants had tuberized.

4.2. Ground cover

Hermes emerged later than M Piper and V Sovereign and ground cover development was therefore delayed. As early as 9 June, early over-watered plots (I+ 0-3) had more advanced ground cover development than I and I- plots (Figure 4.1). This difference was maintained until ground covers approached 100 % but I+ 0-3 plots did not attain complete cover in Hermes and V Sovereign and commenced senescence only 2-3 weeks after maximum cover in all varieties. The canopies of early over-watered plots were almost fully senesced by final harvest in September and the decrease in ground cover was greatest in Hermes and V Sovereign. There was no significant difference in ground cover in Hermes and V Sovereign between any of the other irrigation treatments, even between irrigated and unirrigated plots. There was only one interaction between variety and irrigation regime, indicating that varieties generally did not behave differently to water shortage or excess, but I- M Piper senesced earlier than I, whereas in Hermes and V Sovereign this did not occur. However, over-watering for 3 weeks after tuber initiation was extremely detrimental to canopy survival. A later over-watered period 10-13 weeks after initiation had no effect on ground cover compared with normal full irrigation.

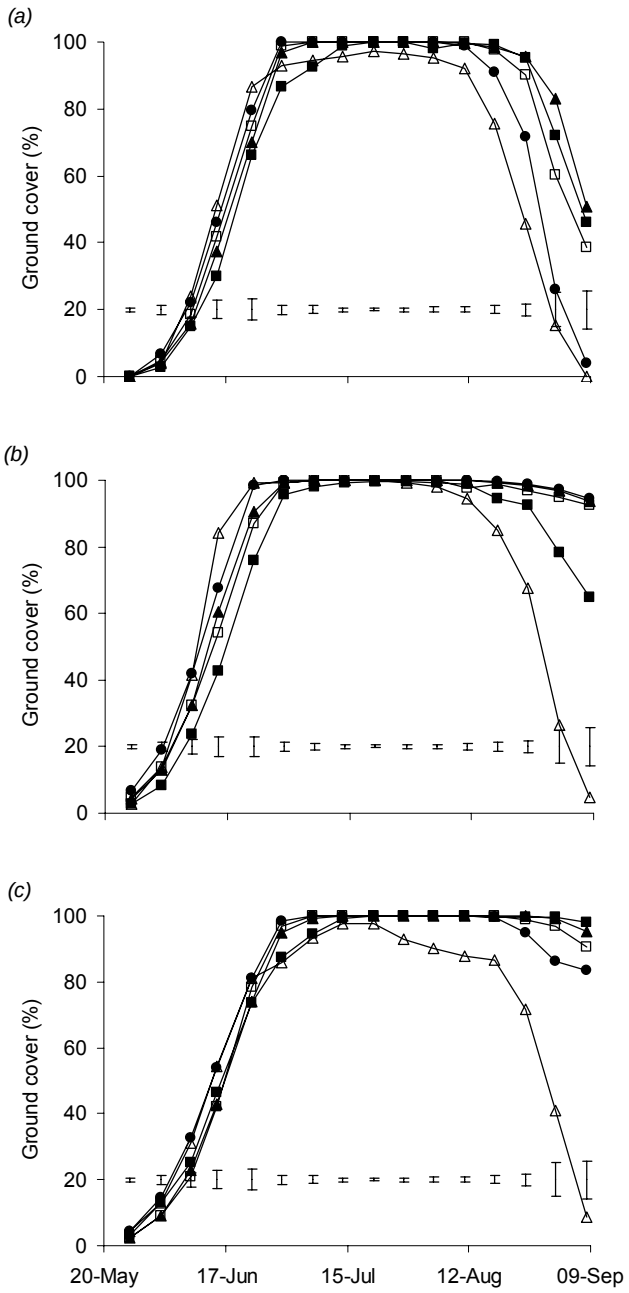

FIGURE 4.1. GROUND COVER IN *(A)* HERMES; *(B)* M PIPER; *(C)* V SOVEREIGN. I-, ■; I, □; I- 4-6, ▲; I+ 0-3, △; I+ 10-13, ●. S.E. BASED ON 27 D.F.

4.3. Soil moisture deficits

4.3.1. Modelled

Reference crop evapotranspiration demand in June and July (3.54 mm/day) was higher than average for these months in Cambridge (3.17 mm/day) but August was average (3.13 mm/day). Deficits in unirrigated crops increased to 50-54 mm in mid-July, then there was almost adequate rainfall for the next 4 weeks to satisfy demand before a dry late August and September caused unirrigated crops to exist on soil reserves, so that by final harvest deficits had reached *c*. 56-60 mm (Figure 4.2). Deficits in irrigated crops averaged 11 mm with a maximum of 25 mm. During the restricted period of I- 4-6 treatments, deficits reached *c*. 45 mm. Deficits during over-watered periods in I+ 0-3 and I+ 10-13 treatments were modelled as zero since the model calculated that excess water would drain away prior to the next irrigation and that the soil water status would not affect root function. Clearly, this was not the case (see section 4.3.2).

4.3.2. Measured

Measurements of soil water content with Theta probes were unreplicated, therefore the values for different treatments should be treated with caution. The soil moisture deficits are based on the top 20 cm of soil only as this was the depth where the probe sensors were located. The probe data indicated that the over-watered treatments were maintained in an over-full status for the entire duration of the period, averaging 7.5, 5.4 and 5.7 mm overfill for Hermes, M Piper, V Sovereign, respectively, for the I+ 0-3 treatment and 6.6, 3.0 and 5.7 mm overfill for the I+ 10-13 treatment (Figure 4.3). Total pore space of the same soil type in an adjacent experiment was typically 60 % at 15-20 cm depth, therefore the air-filled pore space (*c*. 24 %) of these over-filled soils should still have been sufficient for root respiration but this assumes no “plugging” of pores with degraded silt and clay particles. The maximum deficit reached in the ridge (top 25 cm of profile) in irrigated (I) treatments during the scab control period was apparently 6-8 mm, which should have lead to reasonably good scab control based on previous work at CUF (Stalham & Firman 1996).

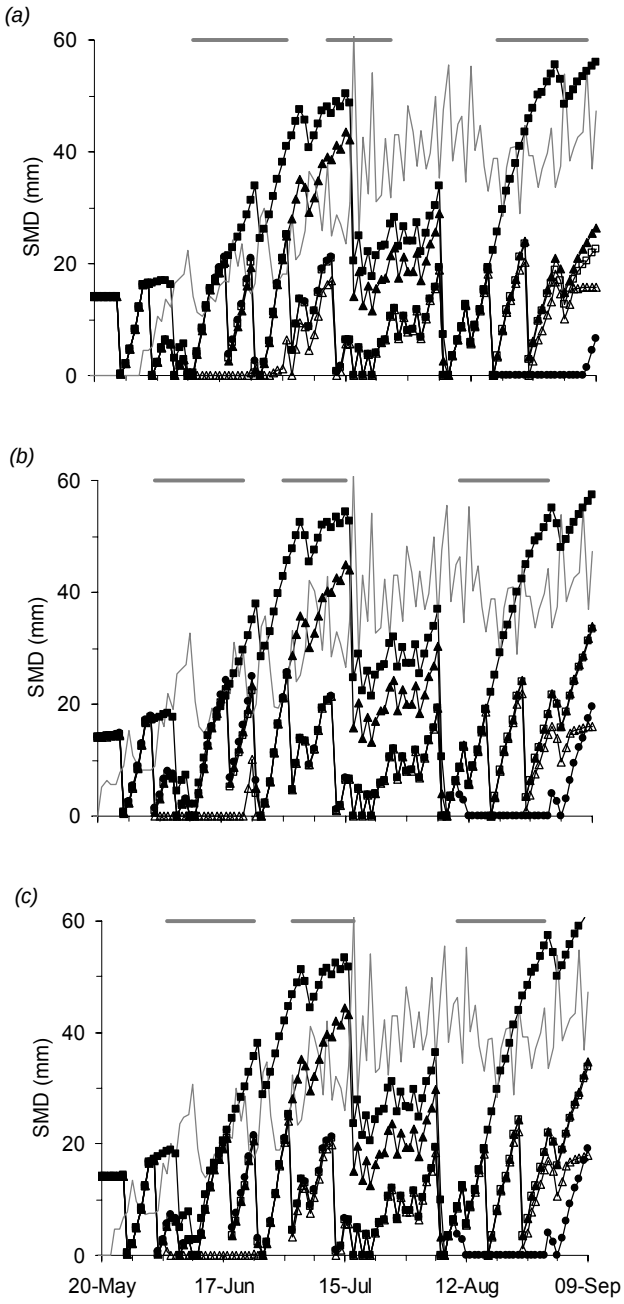

FIGURE 4.2. MODELLED SOIL MOISTURE DEFICITS (SMD). *(A)* HERMES; *(B)* M PIPER; *(C)* V SOVEREIGN. I-, ■; I, □;

I-4-6, ▲; I+ 0-3, △; I+ 10-13, ●. THICK SOLID LINES INDICATE PERIODS OF IRRIGATION RESTRICTION/OVER-WATERING IN I+ 0-3, I- 4-6 AND I+ 10-13, RESPECTIVELY.

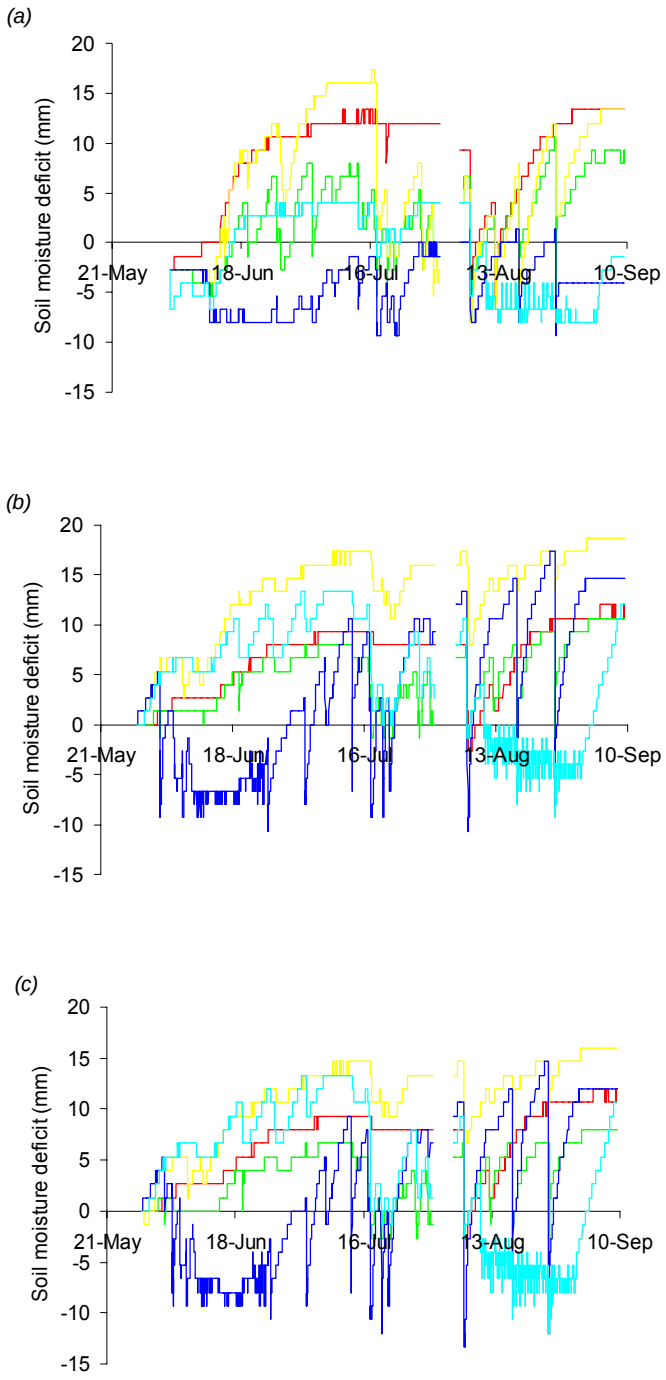

FIGURE 4.3. MEASURED SOIL MOISTURE DEFICITS IN TOP 20 CM OF RIDGE. *(A)* HERMES; *(B)* M PIPER; *(C)* V SOVEREIGN. I-, —; I, —; I- 4-6, —; I+ 0-3, —; I+ 10-13, —.

4.4. Number of tubers and tuber yield

There was a very large increase in the number of tubers in early over-irrigated plots of all varieties that was not caused by variation in the number of stems (Table 4.1). All plots except unirrigated were irrigated for the first time at tuber initiation and canopies were similar across irrigation regimes at this stage. Canopy development was faster in early over-irrigated plots over the next 2 weeks which resulted in a greater light absorption, but this is unlikely to explain the magnitude of the effect. The ground cover in the 2 weeks post initiation in Hermes and V Sovereign was increased in I+ 0-3 treatments in relative terms by 17 % compared with the mean of all other irrigated treatments, whereas in M Piper, there was a 30 % increase. However, the number of tubers in I+ 0-3 plots was 33-40 % greater than I and I+ 10-13 plots. It is uncertain whether the low soil moisture deficits in I+ 0-3 plots aided retention of initiated tubers in the 2-3 weeks post initiation or stimulated an increased number of tubers to be initiated but there were many more tubers in the 20-50 mm grade in I+ 0-3 plots than other treatments. The size of these increases in number of tubers in early over-watered plots compared with normal irrigation has not been seen in previous experiments conducted by CUF.

The highest fresh and dry weight yields were obtained with the “normal” irrigation (I) practice at CUF (Table 4.1). Restricting irrigation to any extent reduced yields significantly. Over-watering in August had little detrimental effect on yield but early over-watering reduced yields considerably, such that the yields were similar to completely unirrigated crops. There is a clear lesson to be learned here in avoiding the temptation to over-irrigate during scab control. Effects of irrigation regime on yield were similar for all three varieties.

Variety	Irrigation regime	No. of mainstems (000/ha)	No. of tubers (000/ha)	Total yield (t/ha)	Yield > 40 mm (t/ha)	Tuber dry matter yield (t/ha)
Hermes	I-	102	405	48.1	46.3	11.1
	I	133	461	63.2	60.9	14.6
	I- 4-6	102	426	58.4	56.1	12.9
	I+ 0-3	101	580	44.7	38.9	10.2
	I+ 10-13	101	413	59.3	57.4	14.0
M Piper	I-	130	470	52.6	49.4	12.5
	I	147	523	66.2	63.0	15.4
	I- 4-6	112	401	57.8	55.6	13.3
	I+ 0-3	144	712	56.7	47.5	11.5
	I+ 10-13	140	491	66.8	64.4	15.1
V Sovereign	I-	163	360	60.5	58.3	12.1
	I	147	414	72.4	69.3	14.5
	I- 4-6	137	351	66.5	64.7	13.7
	I+ 0-3	176	538	58.5	52.7	11.7
	I+ 10-13	171	373	70.4	69.1	14.2
S.E. (27 D.F.)		13.8	43.1	4.07	4.09	0.81

TABLE 4.1. NUMBER OF MAINSTEMS AND TUBERS AND TUBER YIELDS AT FINAL HARVEST

4.5. Common scab incidence and severity

All tubers were infected with common scab but the incidence of tubers with < 5 % surface area infected was lowest in M Piper (43.3 %) and similar in Hermes (73 %) and V Sovereign (81 %). In M Piper and V Sovereign, I+ 0-3 plots had more tubers with slight infection (< 5 % surface area) than other irrigated treatments, with the largest difference being observed in M Piper (Table 4.2), which supports previous work at CUF. In V Sovereign, however, crops which received no irrigation during the 4 weeks after tuber initiation, had the greatest proportion of tubers with only slight infection (< 5 % surface area, SA). All plots of M Piper and V Sovereign received 15 mm of rain at the onset of tuber initiation which may have reduced scab infection in the I- treatment but the effect in V Sovereign may be an anomaly as it is difficult to explain in relation to most previous work at CUF. However, Stalham & Firman (1996) showed that in one season, tubers from plots protected from rainfall and without irrigation had roughly half the severity of scab (3.6 % surface area) of the best sprinkler-irrigated treatments (6.4 %). This could be explained by the soil being too dry for the growth of actinomycetes coupled with a more limited expansion of tubers in dry plots which prevented the spread of scab over the periderm. Since the soil was wetter in the current experiment, at least one of these explanations seems unlikely. The mean surface area infected with scab showed the same trends as for incidence, with unirrigated M Piper having the most severe scab and more frequent irrigation reducing the severity. In V Sovereign, tubers were much less severely affected by scab than in M Piper, but there was still significantly less severe scab where the soil was kept at, or above, field capacity during the susceptible phase rather than allowing the soil moisture deficit to increase to *c*. 20 mm before irrigating. It is important to note that soil moisture deficits in I, I- 4-6 and I+ 10-13 treatments were only *c*. 6 mm at initiation as these plots were irrigated with 21 mm on 2 June. There was no effect of irrigation regime on scab severity in Hermes, even though it developed more scab, on average, than V Sovereign.

		Incidence < 5 % surface area		Severity
Variety	Irrigation regime	%	Ang. trans.	% SA affected
Hermes	I-	76.0	60.9	6.9
	I	72.0	58.5	6.4
	I- 4-6	70.7	57.7	6.4
	I+ 0-3	62.0	53.1	8.4
	I+ 10-13	83.3	67.9	5.0
M Piper	I-	39.3	36.0	20.9
	I	34.0	35.6	13.9
	I- 4-6	30.7	33.0	13.6
	I+ 0-3	68.0	55.8	6.8
	I+ 10-13	44.7	41.2	10.3
V Sovereign	I-	98.7	86.2	2.4
	I	71.3	57.7	6.7
	I- 4-6	76.5	61.6	5.7
	I+ 0-3	88.9	74.0	3.9
	I+ 10-13	68.3	55.8	6.7
S.E. (27 D.F.)			5.21	2.14

TABLE 4.2. COMMON SCAB INCIDENCE AND SEVERITY

The proportions of tubers within the different severity classes are shown in Figure 4. In M Piper, there was a significant increase in the proportion of tubers in the 2-5 % SA class in the I+ 0-3 treatment compared with other irrigation regimes and fewer tubers in the 6-25 % SA category than other irrigated treatments (Figure 4*b*). In V Sovereign, there were significantly more tubers in the 0-1 % SA category and fewer in the 6-25 % SA class in unirrigated than in other irrigation regimes (Figure 4*c*). In Hermes, there were no real differences in the proportion of tubers in each scab category (Figure 4*a*).

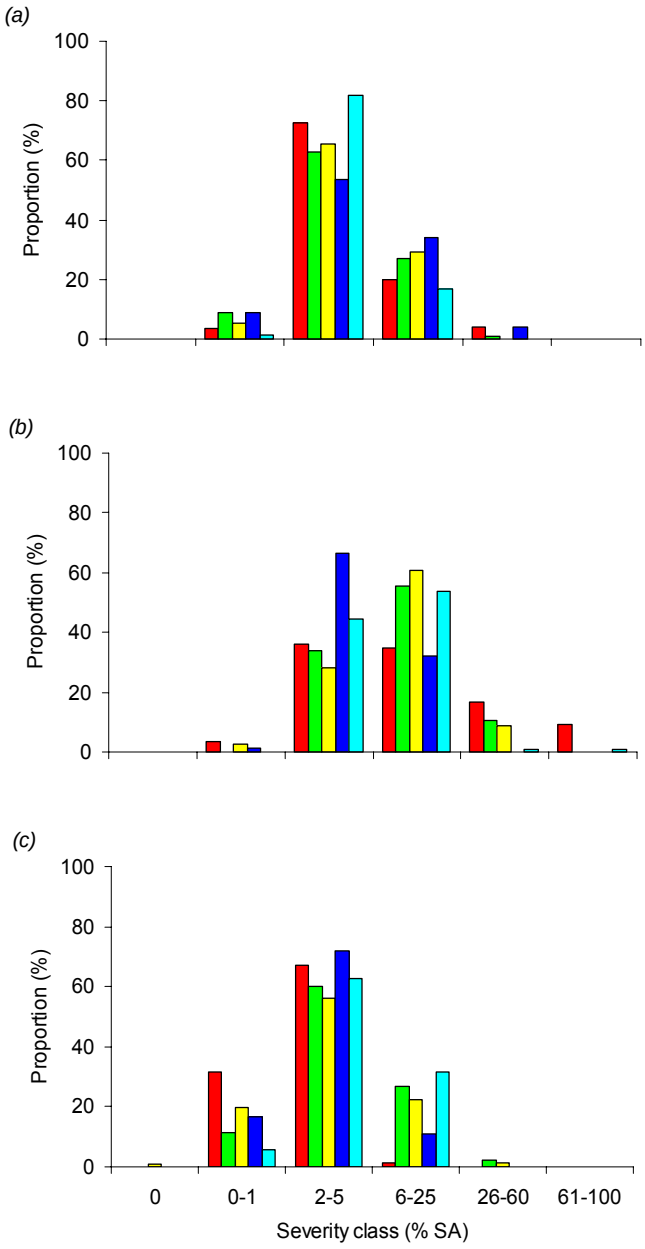

FIGURE 4.4. PROPORTIONS OF TUBERS WITHIN THE DIFFERENT SEVERITY CLASSES (% SURFACE AREA, SA). *(A)* HERMES; *(B)* M PIPER; *(C)* V SOVEREIGN. I-, ■; I, ■; I- 4-6, ■; I+ 0-3, ■; I+ 10-13, ■.

4.6. *Streptomyces* sampling on tubers

Populations of total *Streptomyces* containing the 16S gene sequence together with populations of pathogenic *Streptomyces* that elicit thaxtomin A production in tubers (txt*)* were measured during the 5 weeks after tuber initiation. Populations of 16S were almost undetectable for the first 2 weeks after initiation (Figure 4.5). There was an increase in 16S populations after 3 weeks in I- and I but remained very low I+ 0-3. The largest increase in 16S population was between 3 and 4 weeks after initiation and at this stage I treatments had similar populations to I-. Soil moisture deficits in ridge (0-25 cm depth) had increased to *c*. 17 mm in both treatments by this stage before the I treatments were irrigated 17 days after initiation, which resulted in a large decrease in 16S in I treatments, particularly in M Piper (Figure 4.5).

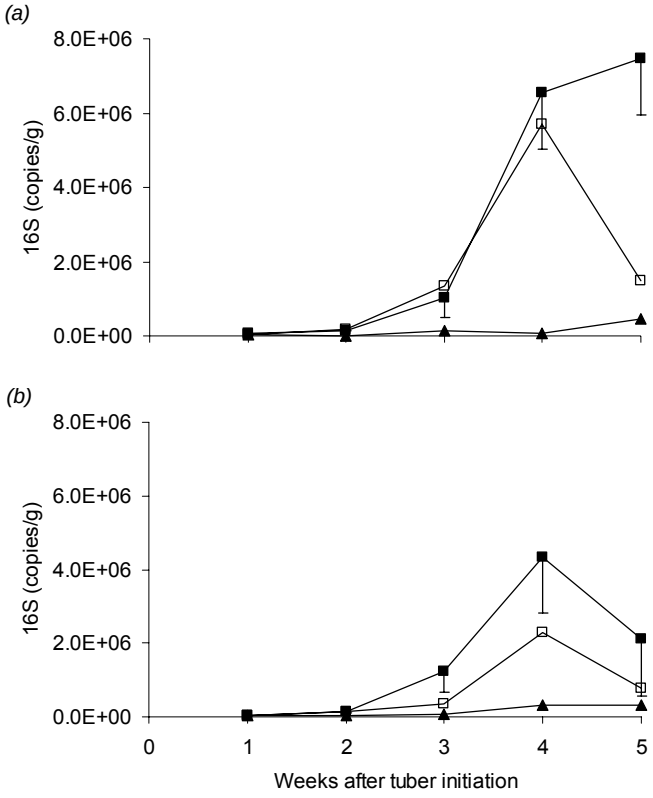

FIGURE 4.5. TOTAL 16S *STREPTOMYCES* POPULATIONS IN *(A)* M PIPER; *(B)* V SOVEREIGN. I-, ■; I, □; I+ 0-3, ▲. S.E. BASED ON 10 D.F.

The populations of pathogenic *Streptomyces* (txt) were undetectable for the first 2 weeks after initiation (Figure 4.6) and remained undetectable in I+ 0-3 treatments in both varieties for the entire 5-week sampling period (except for an extremely low level recorded in V Sovereign 4 weeks after initiation). The populations in I- treatments increased rapidly between 3 and 4 weeks after initiation and remained significantly higher than I and I+ 0-3 for the rest of the sampling period in M Piper and showed a similar, though much less evident change in V Sovereign. In 2007, there was a rapid increase txt *Streptomyces* between 1 and 3 weeks after initiation whereas the largest increase in 2008 was between 1 and 2 weeks after initiation. In dry conditions there appears to be a lag period after tuber initiation before pathogenic *Streptomyces* populations increase.

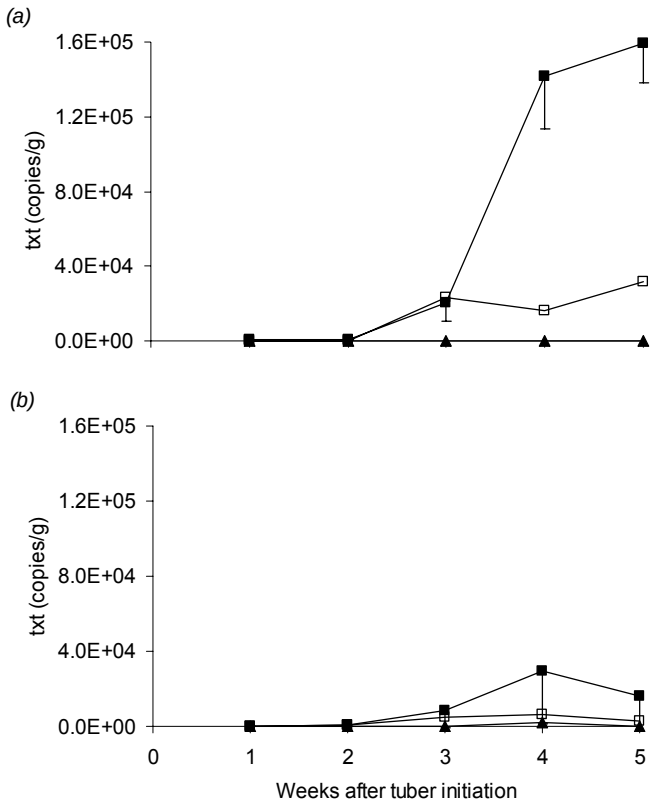

FIGURE 4.6. THAXTOMIN-ELICITING (TXT) *STREPTOMYCES* POPULATIONS IN *(A)* M PIPER; *(B)* V SOVEREIGN. I-, ■; I, □; I+ 0-3, ▲. S.E. BASED ON 10 D.F.

The ratio of txt:16S in M Piper was similar for I- and I until between 3 and 4 weeks after initiation when irrigation appeared to reduce the ratio significantly but the ratio returned to a similar value 5 weeks after initiation despite a difference in SMD of over 30 mm (Figure 4.7). Changes in the txt : 16S ratio over time in V Sovereign were less evident. Owing to the undetectable txt populations in I+ 0-3 treatments, the ratio of txt:16S was zero for both varieties, with the exception of the sampling at 4 weeks after emergence in V Sovereign (Figure 4.7).

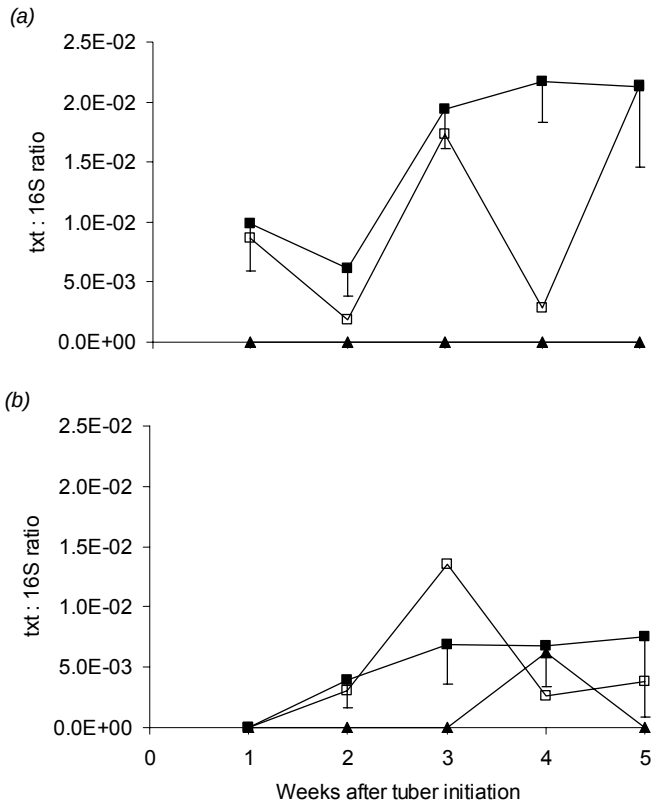

FIGURE 4.7. RATIO OF TXT : 16S POPULATIONS IN *(A)* M PIPER; *(B)* V SOVEREIGN. I-, ■; I, □; I+ 0-3, ▲. S.E. BASED ON 10 D.F.

4.7. Production of DNA sequence libraries for identification of antagonists

Approximately 400,000 DNA sequences have been obtained, representing bacterial diversity in field trials conducted in 2008 at two commercial field sites as well as irrigation trials at CUF (comprising samples collected during the Defra LINK project) as well as the 2009 trials at CUF described in this report. The analyses presented in this report constitute mainly identification of taxa by large-scale comparison of public nucleotide databases with sequence libraries obtained by pyrosequencing.

4.7.1. Bacterial populations in field trials at CUF

Initial analyses were done on DNA samples archived from field trials conducted at CUF in 2008. Approximately 7000 sequences of the bacterial 16S variable region 1 (V1) were obtained from tubers four weeks after initiation in both irrigated and unirrigated plots. Classification of these sequences revealed broad differences between irrigated and unirrigated plots, including an increase in bacteria belonging to the phyla Bacteriodetes and Acidobacteria in irrigated plots (Figure 4.8).

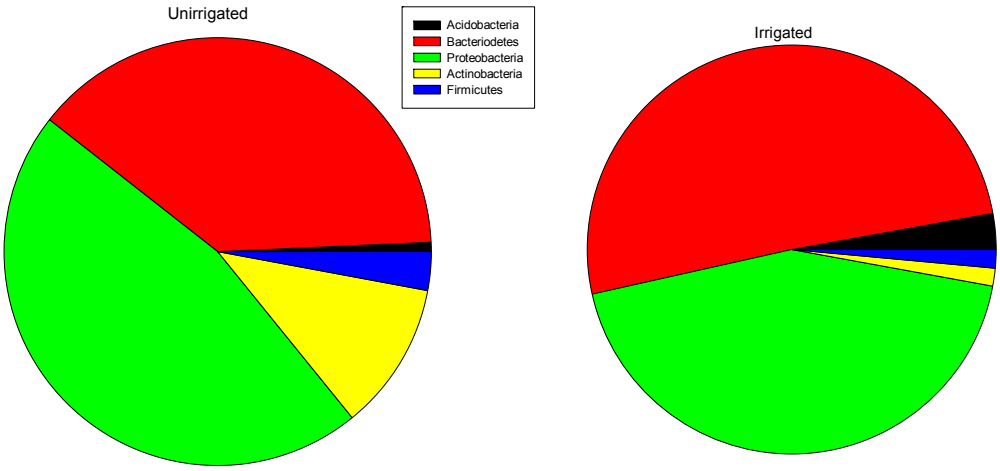

FIGURE 4.8. RELATIVE PROPORTIONS OF THE FIVE MOST ABUNDANT BACTERIAL PHYLA IDENTIFIED ON THE SURFACE OF MARIS PIPER TUBERS, IN IRRIGATED OR UNIRRIGATED PLOTS IN FIELD TRIALS CONDUCTED AT CUF IN 2008. MEASUREMENTS WERE MADE FOUR WEEKS POST TUBER INITIATION.

Similarly, sequences were obtained from the Maris Piper trials at CUF in 2009. Figure 4.9 differences in bacterial flora between irrigated and unirrigated plots. The most significant increases in population as a result of irrigated were observed in the bacterial orders *Flavobacteriales* and *Bacillales*, with smaller increases also observed in *Enterobacteriales* and *Sphingomonadales*. Populations of *Actinomycetales*, which includes the genus *Streptomyces*, some of which are responsible for potato scab, were clearly reduced in irrigated plots compared to unirrigated.

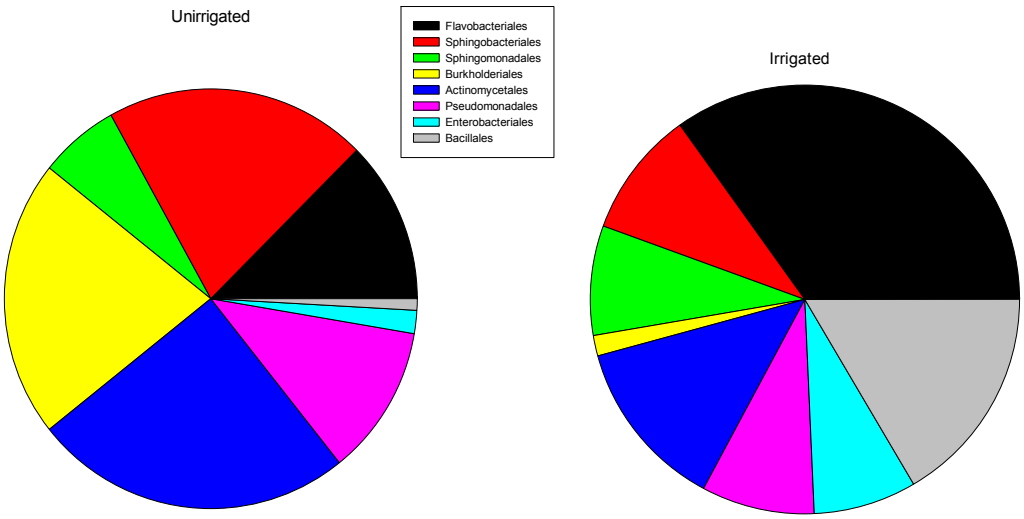

FIGURE 4.9. RELATIVE PROPORTIONS OF THE EIGHT MOST ABUNDANT BACTERIAL ORDERS ON THE SURFACE OF MARIS PIPER TUBERS, IN IRRIGATED AND UNIRRIGATED PLOTS IN TRIALS CONDUCTED AT CUF IN 2009. MEASUREMENTS WERE MADE AT FOUR WEEKS POST TUBER INITIATION.

Levels of *Flavobacteriales* were also greater in irrigated plots of Vales Sovereign compared with unirrigated plots of the same cultivar (Figure 4.10). However, this is the only significant similarity between trials of these two varieties. For example, in Vales Sovereign plots, greater levels of *Methylophilales* and *Caulobacteriales* were apparent in irrigated plots, but levels of *Sphingomonadales* (which was increased in irrigated Maris Piper plots) was lower in irrigated plots.

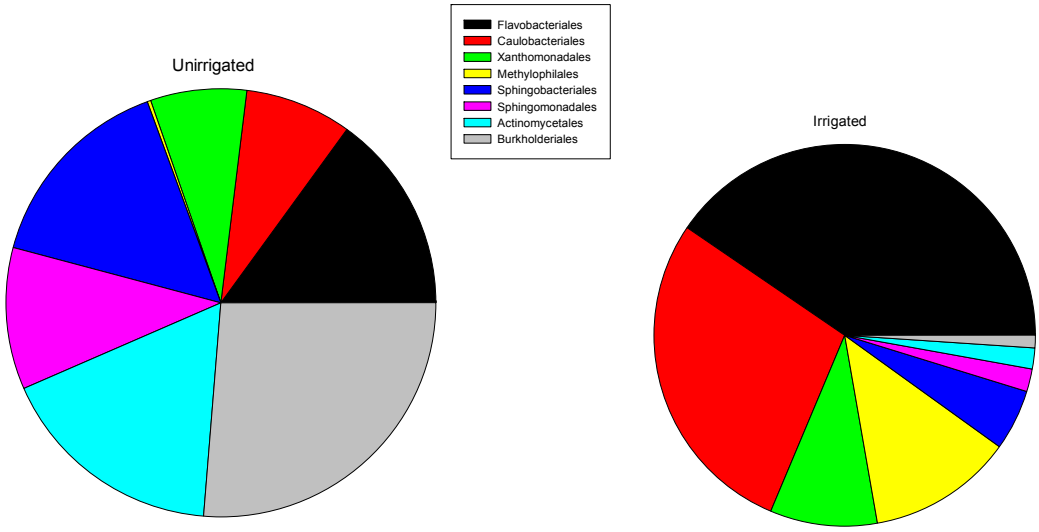

FIGURE 4.10. RELATIVE PROPORTIONS OF THE EIGHT MOST ABUNDANT BACTERIAL ORDERS ON THE SURFACE OF VALES SOVEREIGN TUBERS, IN IRRIGATED AND UNIRRIGATED PLOTS IN TRIALS CONDUCTED AT CUF IN 2009. MEASUREMENTS WERE MADE AT FOUR WEEKS POST TUBER INITIATION.

Changes in bacterial populations in irrigated and unirrigated plots can best be illustrated by plotting relative levels of bacterial orders during the period in which scab control by irrigation is most effective. Figure 4.11 shows relative levels of the same 12 bacterial orders in Maris Piper and Vales Sovereign plots. While an increase in *Flavobacteriales* in irrigated plots is apparent in irrigated plots of both cultivars between weeks two and four, other changes in populations, though they may be significant within plots, are not shared between cultivars.

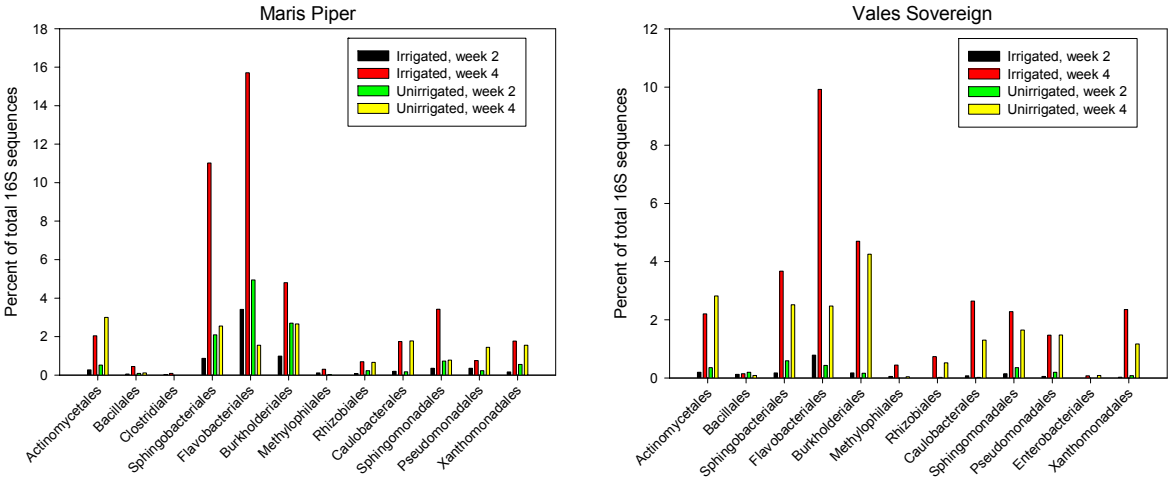

FIGURE 4.11. CHANGES IN POPULATION OF 12 BACTERIAL ORDERS IN MARIS PIPER AND VALES SOVEREIGN

Our initial proposal was to use amplicon pyrosequencing on a small number of sites to identify and produce sequence from key taxa whose populations were shown to increase in response to irrigation. The high cost of pyrosequencing would have made extensive sequence analysis of multiple sites too expensive, so we had planned in subsequent experiments to substitute sequencing with quantification of selected microbial taxa by real-time PCR to give an indication of the changes in levels of specific organisms in irrigated and unirrigated fields. However, our first sequencing experiments showed that population changes may vary significantly between years, even when under similar irrigation conditions, so selecting specific taxa from one trial to study in another would be unlikely to yield meaningful results. A more informative strategy would be to produce sequence data from a wider variety of field sites, thus giving a more comprehensive picture of population fluxes at different locations under different treatments. During the course of this project, Fera invested in pyrosequencing technology, and is now able to perform the method in house. This made pyrosequencing more cost effective, and with a small extra investment from some Fera 'seedcorn' funds we were able to sequence a wider range of samples from the trials conducted at 2008 at CUF as well as commercial fields. The data constitutes the most comprehensive picture yet of microbial populations in UK potato fields under known conditions.

4.7.2. Bacterial populations in commercial fields

During the previous Defra LINK project, a set of trials were established in commercial potato fields to determine the effect of location and seed source on *Streptomyces* populations and scab levels. DNA samples from these trials were archived at Fera and used in this study to examine whether population changes observed in the controlled trials at CUF were also apparent in commercial fields. We chose to use DNA sequencing rather than real-time PCR as the latter is a targeted approach and our observations on the diversity of response to irrigation suggested that specific amplification of a restricted set of taxa would not give a true representation of diversity in the field.

Bacterial 16S amplicons were generated from trial material from five commercial sites where two stocks of Maris Piper seed were grown. Scab levels and *Streptomyces* populations had previously been determined (Fig 4.12).

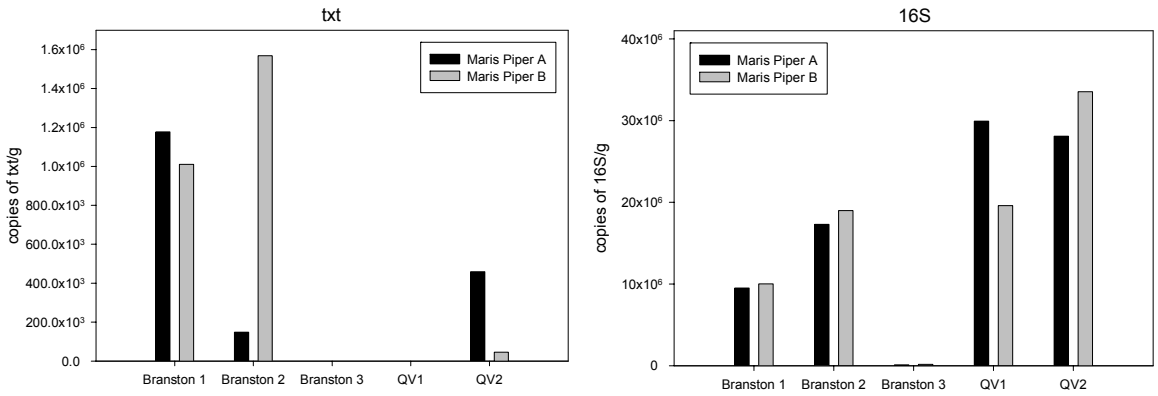

FIGURE 4.12. LEVELS OF PATHOGENIC *STREPTOMYCES* (*TXT*) AND TOTAL ACTINOMYCETES (16S) THREE WEEKS AFTER TUBER INITIATION IN COMMERCIAL FIELD TRIALS IN 2008.

Previous sequencing experiments had indicated that the order *Flavobacteriales* showed increased abundance in irrigated soils where scab levels were lower. Levels of *Flavobacteriales* in the commercial trials are shown in Figure 4.13. These trials were conducted using two different sources of Maris Piper seed. Populations of all major microflora taxa found on the surface of thee tubers were similar on plants grown from different seedstocks when they were planted in the same field. This is illustrated in the populations of *Flavobacteriales* (Figure 4.13) although other taxa, when examined, showed the same phenomenon (data not shown).

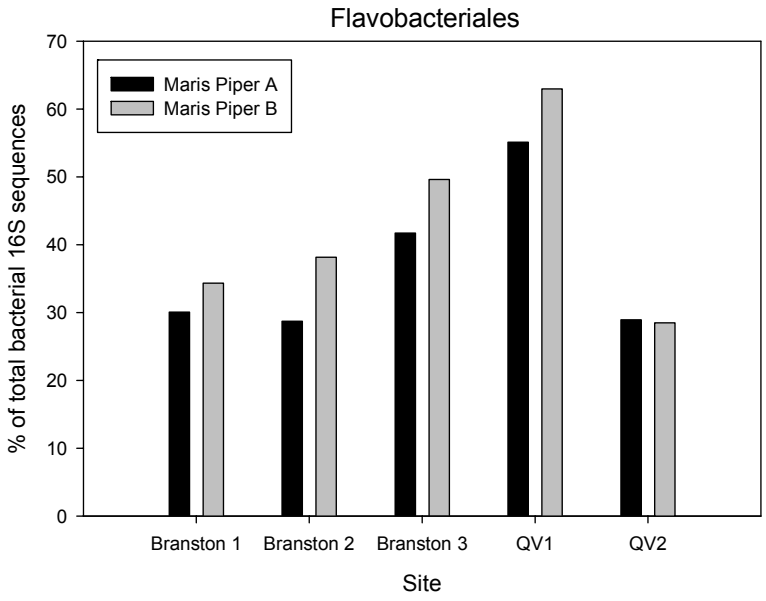

FIG 4.13. LEVELS OF THE BACTERIAL ORDER *FLAVOBACTERIALES* IN TUBERS HARVESTED FROM COMMERCIAL FIELD TRIALS THREE WEEKS AFTER TUBER INITIATION. FIELDS WERE PLANTED WITH TWO SEED STOCKS, MARIS PIPER A AND B.

4.7.3. Fungal and archaeal populations

DNA sequence data was also obtained from PCR amplicons specific to fungi (ITS) and archaea (16S). Archaeal diversity in all plots was low, although the sequencing suffered from cross reactivity of primers with plant plastid sequences. These results are not shown. There was also some cross-reactivity between fungal ITS primers and plant sequences, although fungal sequences were obtained. All fungi identified belonged to the phylum Ascomycota. Figure 4.14 shows relative abundance of fungi detected in Maris Piper in irrigated and unirrigated plots at CUF in 2009, four weeks after tuber initiation. Populations in unirrigated plots were dominated by *Fusarium* spp., whereas diversity was higher in irrigated plots.

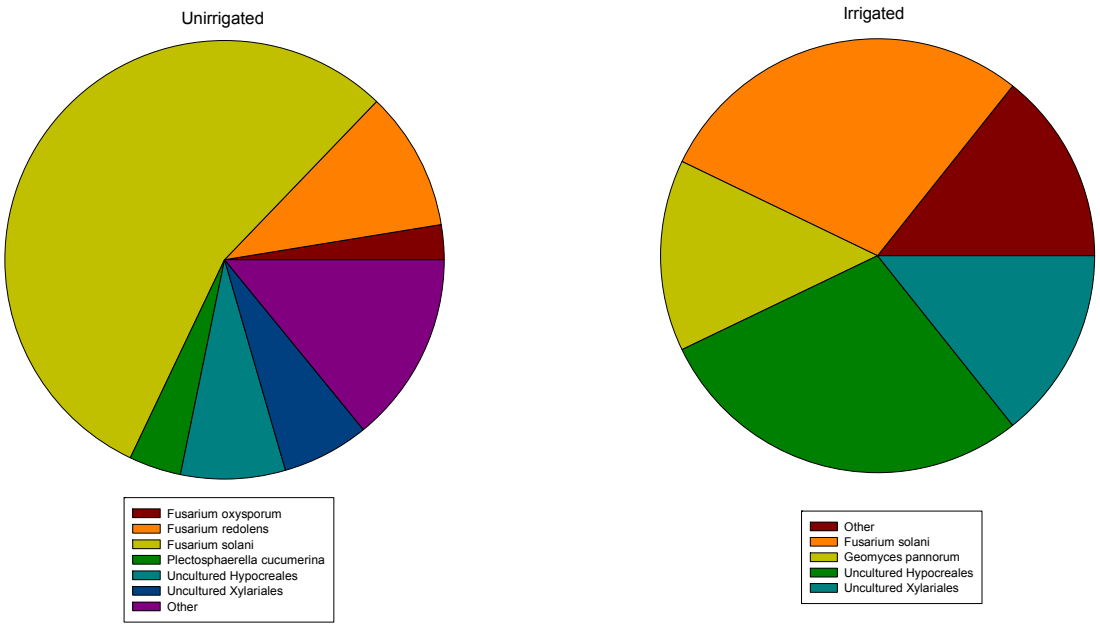

FIGURE 4.14. RELATIVE FUNGAL POPULATIONS IN MARIS PIPER IN IRRIGATED AND UNIRRIGATED PLOTS AT CUF IN 2009, SAMPLES FOUR WEEKS POST TUBER INITIATION.

5. DISCUSSION

Data from three field experiments, including those described here and experiments conducted under the previous LINK project in 2007 and 2008, indicate that the critical period in which scab control can be effected by control of soil moisture, is between one and three weeks after tuber initiation.

Our other observation from the current experiments, that V Sovereign developed smaller populations of pathogenic *Streptomyces* than M Piper, is also interesting. Adams & Lapwood (1978) found that microbial development appeared to be similar on susceptible M Piper and relatively resistant Pentland Crown, suggesting that differences in resistance in their experiments were not caused by modifications to the surface microflora. In the current experiment, V Sovereign developed significantly lower populations of pathogenic *Streptomyces* than M Piper in dry soils but similar populations in irrigated soils which suggests that disease suppression in resistant varieties may indeed be combination of reduced pathogen loading as well as possible changes in lenticel differentiation and susceptibility to invasion by *Streptomyces*. However, our ability now to analyse total microbial populations in great detail means that changes to microflora on the surface of tubers, which may not have been evident in comparisons made using traditional cultural methods, are more readily detected.

Our previous work demonstrated that scab levels in commercial fields are influenced more by the location than the level of scab on seed. This is presumably because suppressive factors, including antagonistic soil microbiota, vary significantly between sites. Our sequencing data supports this, as we have yet to identify microbial taxa that are consistently linked to suppression of scab in the field. Thus suppression in one soil may be mediated by a different set of microbial flora than another soil. These observations bear out the value of our pyrosequencing approach to characterise microbial populations. A more targeted method, such as real-time PCR quantification of specific taxa, would not have identified population changes evident in the diverse samples analysed in this study. Moreover, other untargeted methods, such as community fingerprinting protocols often employed in this type of study, do not readily allow identification of taxa with the accuracy of sequence-based identification.

Our most informative results were obtained for bacteria. This is primarily because a wealth of sequence information exists for conserved bacterial sequences on which we were able to base PCR primer design and which could interrogate to identify taxa from which sequences originated.

In a smaller scale study, we had previously determined that certain bacterial taxa increased in population in response to irrigation. Most notable was *Pseudomonas*, sequences from which were up to ten times more abundant in irrigated plots (in which scab levels were observed to be lower) than in unirrigated plots. Where differences were observed between the irrigation regimes examined in this study, other bacterial taxa were shown to undergo population changes. The only bacterial taxon that increased in abundance in irrigated soils in more than one trial was *Flavobacteriales.* We consider this significant as this was observed in controlled trials at CUF on two varieties (Maris Piper and Vales Sovereign) in 2009 as well as in commercial field sites in 2008. These latter, commercial, sites were not subject to the same controlled irrigation regimes but were shown to differ markedly in scab level and *Streptomyces* populations. Indeed our previous study showed that location (and by implication, the soil type) was the most influential factor in scab development in commercial field sites.

Higher levels of *Flavobacterium* were observed in fields that had lower scab and fewer pathogenic *Streptomyces* sequences. This group contains organisms able to secrete biosurfactants, a known mediator of biological control (Bodour et al 2003). However, the population structure of *Flavobacteriales* in the various plots in which these organisms were observed was not necessarily the same, and further sequencing work would be necessary to determine this.

Our observation that populations of the major soil microflora taxa were similar on tubers derived from two different seedstocks grown in the same field suggest that, as we have also observed for levels of *Streptomyces*, it is the endemic soil microflora which exerts the greatest influence on microbial populations on the surface of tubers, irrespective of populations in seed.

The wealth of sequence data produced by our pyrosequencing approach means that datasets are large and complex, and lend themselves to many forms of analysis. Many of these analysis tools are novel and developed for specific data. Thus our analysis of this data will continue. Tools for analysis of fungal sequences, for example, are not as advanced as those for bacterial 16S sequences. However, the data is already raising key questions regarding our understanding of how soil microbial communities can suppress disease. For example, are there common phenotypic traits between the microbes suppressing disease in different soils? It is possible that these traits may cross traditional taxonomic boundaries, since gene exchange is likely to occur in the complex microbial community of the soil. Further work on identification of potential antagonists needs to develop ways to recognise these potential phenotypic commonalities as well as DNA-based identification of taxa.

6. CONCLUSIONS

Watering according to the CUF Irrigation Scheduling model resulted in the highest yield and any restriction to irrigation was detrimental to yield. Control of common scab was generally better in two packing varieties where soils were kept at field capacity following tuber initiation than where irrigated at larger (e.g. 20 mm) soil moisture deficits or unirrigated. However, over-watering during this period had large negative effects on canopy duration, yield and tuber cracking (data not shown) compared with less frequent irrigation. The observation of an apparent lag period after tuber initiation before pathogenic *Streptomyces* populations increased in dry conditions is useful new information since it is widely believed that soils must be wet at initiation to prevent common scab. However, the data from three experiments over the period 2007-2009 show that the critical period for control of soil moisture is between 1 and 3 weeks after initiation. This needs to be investigated more closely before changes can be confidently made to current recommendations which suggest maintaining wet soils from initiation for 3-4 weeks.

Measurement of microbial populations by pyrosequencing indicated a diversity of dominant taxa in samples from different sites. It was not possible to identify microorganisms consistently linked with low levels of scab either at the species or genus level. However, higher-level taxa have been identified which are promising candidates for further investigation as potential antagonists to pathogenic *Streptomyces*. Further research is necessary to determine how much (if any) of the effect of water on scab suppression is a direct effect on *Streptomyces* and how much is due to increases in antagonists. Also, it is important to discover which fractions of

the soil microflora identified in this study are antagonistic to pathogenic *Streptomyces*, either in the laboratory or the field.

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8. ACKNOWLEDGEMENTS

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