Project title:	Development of an early warning test indicating the risk of Cavity Spot in Carrot. Phase 2: Validation
Project number:	FV 448a
Project leader:	Dr Nathalie Verhoef, NSure
Report:	Final report, 24-11-2017
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Date project commenced:	01-03-2017
Expected completion date:	31-10-2017

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The results and conclusions in this report are based on an investigation conducted over a two-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

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

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GROWER SUMMARY

Headline

Prevent losses caused by *Pythium violae* by using a practical molecular test determining the risk of cavity spot in carrots.

Background

Cavity spot is a major disease in the UK and is mainly caused by *Pythium violae*. Cavity spot reduces harvest quality. Cavity spot carrots are not acceptable for packing, but are used at low levels in processing. Severe infections (either high incidence or deep lesions), would be rejected from the processing market.

An early indication for cavity spot would be of great value, as it can be used as a decision support system. The test has to assess risk on cavity spot at two cost adding moments: before distribution of straw and before fields are covered. Selecting low risk fields will reduce losses and leads to less costs for labour and straw.

Development of diagnostic methods enabling early detection of cavity spot would be of great importance in limiting the economic losses. Assays have been developed to detect *Pythium* species in the soil or in carrot tissue (White *et al.*, 1996; Klemsdal *et al.*, 2008; Barbara D.J. *et al.*, 2010). However, a positive result on *Pythium violae* in the soil or on carrots is no guarantee that cavity spot will occur as the vitality status of the carrot plays an important role too. To predict whether a certain field of carrots will develop cavity spot, it is necessary to look at the crop status.

The aim of this project was to identify cavity spot specific indicator genes from the most prominent carrot cultivar in the UK ‘Nairobi’. These genes would be used to develop a practical test that quantifies the expression of those genes to determine the risk of cavity spot at an early stage.

Summary

The intended time to develop a molecular test to detect cavity spot at an early stage using carrot specific genes was expected to take two years.

Year 1

In collaboration with carrot growers Poskitt, Strawson and seed company Elsoms Seeds, NSure collected samples from the carrot variety ‘Nairobi’ in September and October 2015 from various fields in Nottinghamshire and Yorkshire. Based on the quality evaluation results, NSure selected two fields that showed a low occurrence of cavity spot and two fields with a high occurrence. Frozen samples collected from those fields were studied in detail by RNA

sequencing (RNA-Seq). By using this method, NSure was able to examine the activity of all genes. By comparing the low risk samples to the high risk samples, a longlist of potential indicator genes was created that could be suitable to predict the occurrence of cavity spot. A set of putative indicator genes were scrutinized by testing their predictive power on samples collected from other fields by quantitative RT PCR (qPCR). Several potential indicator genes, survived this testing phase, although it should be mentioned that correlation between the gene expression profiles of the genes that surpassed the testing phase to the occurrence of cavity spot was not perfect. The limited sample collection as well as the low number of quality assessments made it difficult to value the results.

Year 2

In the second year of the project, the putative indicator genes were validated in a new sample-set. Like previous year, samples were collected from various fields in Yorkshire and Nottinghamshire growing Nairobi. In parallel, samples were collected from two trials in which 6 carrot varieties intentionally were infected with *Pythium violae*, *Pythium sulcatum* or a control treatment. Based on the inoculation trials, it could be determined whether the potential indicator genes responded in a similar way in other varieties. Furthermore, it could be studied if *Pythium violae* and *Pythium sulcatum* alter the expression of the potential cavity spot indicator genes in a similar manner. Like previous year, it turned out that it was quite difficult to obtain a reliable sample collection and sufficient quality evaluation results. A complete sample-set was obtained from the commercial fields in Nottinghamshire, but in the fields also a lot of other pathogens were present that complicated the validation. Due to circumstances, a complete sample-set from the commercial fields in Yorkshire could not be obtained. In case of the inoculation trials several complications were encountered. Based on the control treatments, it could be deduced that there was already *Pythium* present in the fields. Furthermore, the occurrence of cavity spot was highly variable between the replicates as well as the presence of other pathogens disturbed the quality evaluation and also the validation.

Regarding the inoculation trial performed on the premises of Elsoms Seeds, no significant differences were observed in susceptibility between the tested varieties towards *Pythium sulcatum* and *Pythium violae*. In the other inoculation trial, a significant difference was observed between Nerac and Nairobi in the presence of *Pythium sulcatum* (Figure 1). Although not significant, this trend was also observed between Nerac and Nairobi in the control and *Pythium violae* treated plots. Nairobi seemed to be more susceptible than Nerac. Based on the severity scale, Nipomo was also more susceptible in the presence of *Pythium sulcatum* than Nerac. Within this trial, there appeared to be a trend that Nerac, Norfolk, Norway had a similar tolerance level against cavity spot whereas Nipomo, Newark and Nairobi seemed to be the more susceptible varieties. The results were similar to those

obtained in a cavity spot field trial performed by Bejo Seeds in the Netherlands in 2015 (personal communication).

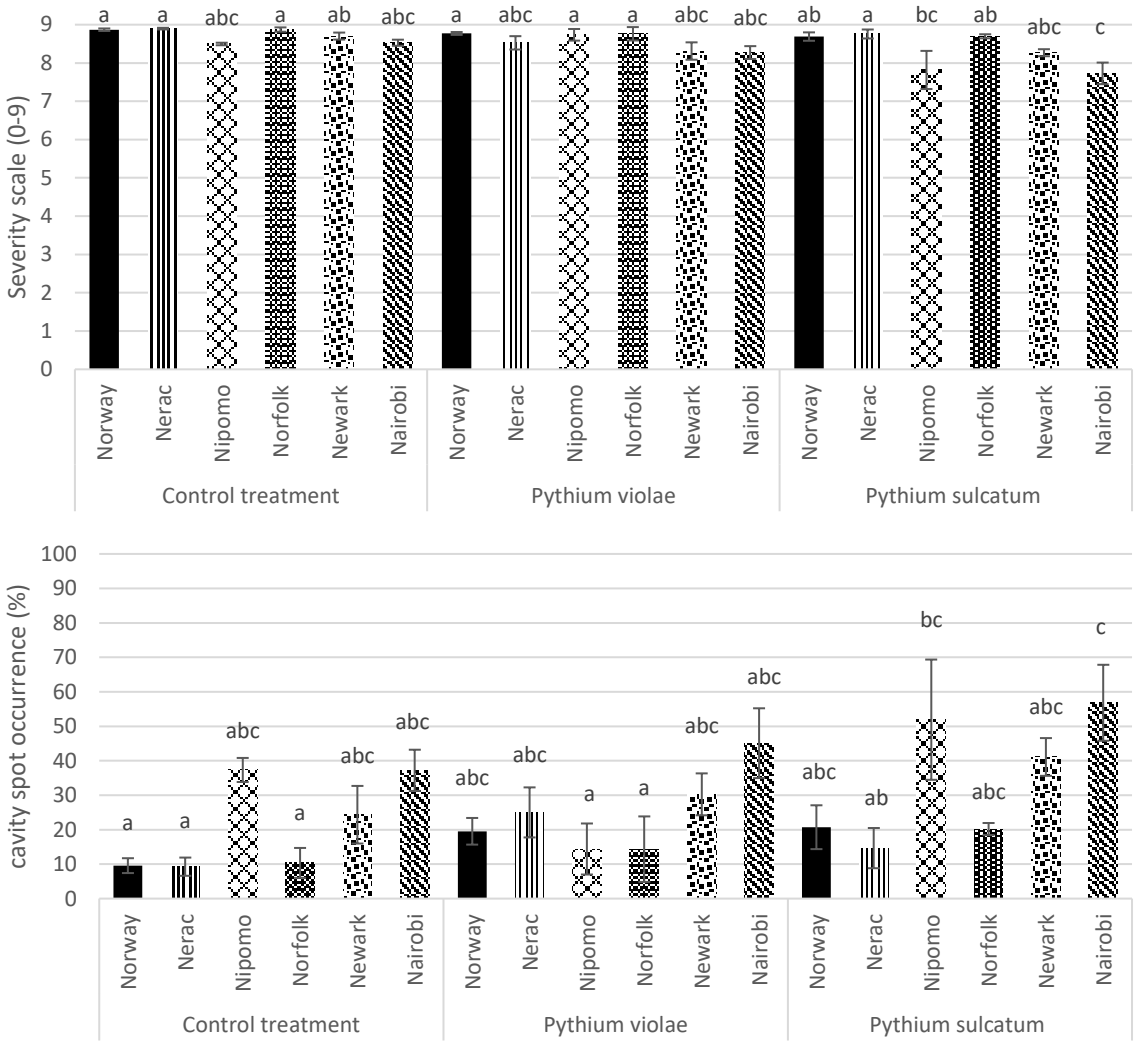

Figure 1. Cavity spot measurements conducted on the inoculation trial performed in Nottinghamshire. Top) Severity scale which ranges from 0 to 9; with 9 no symptoms observed and 0 very severely infected. Bottom) Cavity spot occurrence based on the total number of infected carrots (%). Measurements were conducted in November 2016. Each bar represents the mean based on four repetitions. The error bar represents the standard error. Bars with different letters are significantly different (Tukey's HSD, $p<0.05$).

The expression of the potential indicator genes, was first checked in the frozen samples collected from the commercial fields in Nottinghamshire 2016. Several genes were discarded due to a poor correlation to the occurrence of cavity spot and towards each other. Eleven genes surpassed this validation. Interestingly, 5 out of 11 genes were predicted to be associated with ethylene signalling and 3 other genes seem to respond to (a)biotic stresses. The plant hormone ethylene is involved in many developmental processes, including plant-

pathogen interactions. Root invasion by Pythium spp. is characterized by degradation of host cell walls and plants may respond actively to a Pythium invasion by thickening and lignification of the wall. Ethylene has shown to alter lignification, cell wall synthesis and cell wall composition (Geraats et al., 2002). Furthermore, ethylene insensitive tobacco and Arabidopsis mutants showed increased susceptibility to Pythium spp. (Geraats et al., 2002).

In Figure 2, the gene expression profiles are shown of 5 potential indicator genes and they show a quite similar gene expression pattern towards each other. For the majority of the commercial fields in Nottinghamshire, the gene expression profiles were quite comparable irrespective of the time (August/October) they were collected. For certain fields, variation was observed between the replicates or time points demonstrating that there may have been some heterogeneity within the field.

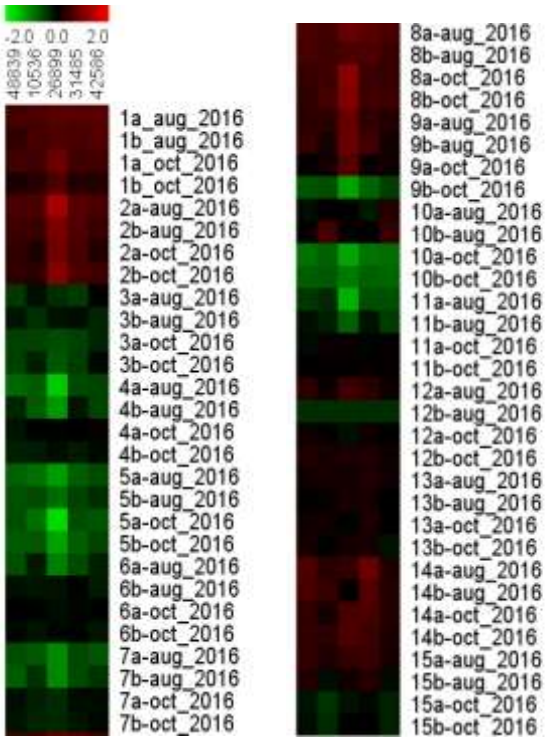

Figure 2. Heatmap representing colour-coded expression levels of 5 potential cavity spot indicator genes collected from commercial fields in Nottinghamshire. Low expression of the gene is indicated in red, high expression in green. The numbers indicate field numbers, a/b the replicates and aug/oct samples collected in August/October.

Regarding the commercial fields in Nottinghamshire, NSure was not able to identify all the high risk fields based on the gene expression patterns of the potential indicator genes. Although the correlation of the potential indicators to the occurrence of cavity spot was not all-decisive, the genes still looked promising especially regarding the predicted function. The set of genes were further validated in samples collected from commercial fields in Yorkshire

and from the inoculation trials. For most samples measured, similar gene activity profiles were observed between the genes as was observed for the Nottinghamshire samples. Nevertheless, correlation of the gene expression profiles of the potential indicators with the occurrence of cavity spot was low. This questions whether these genes are truly cavity spot indicators. The RNA-Seq study performed in the first year was re-evaluated again to find new indicator genes. Some new potential genes were identified, but in the end they did not pass the qPCR validation.

This project made clear that it is difficult or perhaps impossible to find specific genes for cavity spot. It could be that there are no specific genes that are solely altered upon a *Pythium* infection. On the other hand, it could be that the variability within our sample-set complicated the identification of specific genes. A different approach could possibly lead to specific indicator genes. An inoculation trial with *Pythium violae* (mainly found in the UK) should be set up under tight controlled conditions (greenhouse) to assure the absence of pathogens and subsequently RNA-Seq should be performed to identify genes that are altered upon a *Pythium violae* infection. After identification, the potential indicator genes should be monitored tightly in the field in combination with other measures over the years to find genuine patterns and cavity spot indicator genes.

Financial Benefits

Carrot is one of the major crops in the UK. The total cultivated area exceeds 9000 ha 60% of the acreage, approx. 5500 ha, is stored under straw. One hectare results on average in a gross income of £8000. This means that the total turnover of covered carrots is approx. £44million.

Losses due to cavity spot vary between years and geographical regions. Till recently, Scotland for example, had no occurrence of cavity spot. Other regions have more severe problems. In some fields the damage exceeds 40%. On average, cavity spot destroys 3 - 7% of the yearly yield, resulting in a loss between £1.25 and £3million. However, this percentage seems to increase over the years. In 2014 for example, the percentage was estimated to be between 5 and 10%, which almost doubled the losses.

The average cost of covering consist of straw (£3000 per ha) and logistics (transport and covering). In total the costs for covering are approx. £4000 per ha. It is clear that a high cavity spot occurrence means that a grower will not earn (instead: will lose) money on those batches. A predictive test that determines high risk fields, will support a grower to pick only low risk fields for covering.

A predictive test would make UK carrot industry much more profitable in picking the ‘safest’ crops to store over the shorter or longer term.

Action Points

There is no clear change of practice for the growers as no reliable indicator genes were identified within this project.

SCIENCE SECTION

Introduction

Cavity spot is an important limiting factor in the carrot production worldwide. Multiple *Pythium* species, that differ from region to region, are involved in cavity spot. Cavity spot in the UK is mainly caused by *Pythium violae*. Cavity spot is characterised by small sunken elliptical lesions that appear on the tap root, while the aboveground plant parts do not show any visible symptoms. Currently, growers try to minimise the risk on this disease by using cultural practices such as avoiding fields with a history of cavity spot, growing carrots on raised plant beds and the usage of fungicides.

Development of diagnostic methods enabling early detection of cavity spot would be of great importance in limiting the economic losses. ELISA assays as well as PCR-based tests have been developed to detect *Pythium* species in the soil or in carrot tissue (White *et al.*, 1996; Klemsdal *et al.*, 2008; Barbara *et al.*, 2010). However, a positive result on *Pythium violae* in the soil or on carrots is no guarantee that cavity spot will occur as the vitality status of the carrot plays an important role. To predict whether a certain field of carrots will develop cavity spot, it is necessary to look at the crop status.

In 2013, NSure, launched a molecular test that can predict whether a batch of harvested carrots will develop black spots during cold storage (www.nsure.eu). Already months before symptoms become visible, NSure measures the expression of multiple disease related carrot genes that are altered upon an early infection with one of the black spot fungi. To find such indicator genes, NSure uses RNA sequencing (RNA-Seq). By using RNA-Seq, NSure can examine the expression of all genes present in any crop of interest and select those genes in which the expression is linked to a particular trait (Stattin *et al.*, 2012; Kromwijk *et al*., 2013).

The aim of this project was to identify cavity spot specific indicator genes from the most prominent carrot cultivar in the UK ‘Nairobi’ in order to develop a practical test that determines the risk of cavity spot at an early stage. An early indication for cavity spot would be of great value, as it can be used as a decision support system. The test has to assess risk on cavity spot at two cost adding moments: before distribution of straw and before fields are covered. Selecting low risk fields will reduce losses and lead to less costs for labour and straw.

Within the first year of the project (Verhoef, 2016), a set of potential indicator genes for cavity spot have been identified by using RNA-Seq. The search for those genes is extensively described by Verhoef, but also in brief below.

In collaboration with carrot growers and a seed company (Poskitt, Strawson and Elsom Seeds), NSure collected samples from the most prominent carrot variety ‘Nairobi’ in September and October 2015 from various fields in the UK. Poskitt and Strawson evaluated

the fields on the occurrence of cavity spot and the occurrence of cavity spot ranged from 0% to 65%. Based on the quality evaluation results, NSure selected two fields that showed a low occurrence of cavity spot and two fields with a high occurrence. Frozen samples collected from those fields were studied in detail by RNA sequencing (RNA-Seq) enabling NSure to examine the activity of all genes. By comparing low and high risk samples, NSure was able to create a longlist of potential indicator genes that could be suitable to predict the occurrence of cavity spot. Putative indicator genes were scrutinized by testing their predicting power on samples collected from other fields by using qPCR. Several putative indicator genes survived this testing phase. As expected, many of the genes are known to be involved in defence related processes.

Table 1. Objectives and milestones first year

Objective 1.	Collection of carrot samples from different fields in two different areas (Yorkshire and Nottinghamshire) from the largest commercial variety, Nairobi.
Date	**Milestone**
01/11/2015	1.1 First collection of carrot samples (Nairobi) gathered from different sites.
01/05/2016	1.2 Quality evaluation of the sampled plots described for milestone 1.1.
Objective 2.	Selection of a longlist of carrot genes that may determine the risk of development of cavity spot in the variety Nairobi.
Date	**Milestone**
01/09/2016	2.1 A longlist of candidate genes identified by RNA-Seq.
Objective 3.	Validation of carrot indicator genes in Nairobi.
Date	**Milestone**
01/10/2016	3.1 qPCR validation on the carrot collection described for milestone 1.1.
Objective 4.	Continuous and active two-way communication between growers and researchers.
Date	**Milestone**
Continuous	4.1 Share results with growers and other researchers

In the second year, the putative indicators were validated in a new sample-set. As in the previous year, samples were collected from various commercial fields in Yorkshire and Nottinghamshire growing Nairobi. In parallel, NSure collected samples from two trials in which 6 carrot varieties were intentionally infected with *Pythium violae*, *Pythium sulcatum* or a control treatment. Based on the inoculation trials, it could be determined whether the potential indicator genes responded similarly in other varieties. And furthermore, it could be investigated if *Pythium violae and Pythium sulcatum* alter the expression of the potential cavity spot indicator genes in a similar manner. After validation, the aim was to select a final set of indicators and define the decision criteria that would be used to determine whether a batch shows a certain risk at developing cavity spot. In addition, a “grower friendly” sampling protocol would be developed.

The objectives and milestones to be achieved for the second year are depicted in Table 2.

Table 2. Objectives and milestones second year

Objective 1.	Validation of carrot indicator genes in the most important carrot variety, Nairobi
Date	**Milestone**
30/04/2017	1.1 Quality evaluation of the samples plots described for milestone 1.1
30/06/2017	1.2 qPCR validation on the carrot collection described for milestone 1.1
Objective 2.	Validation of carrot indicator genes in various samples collected from 6 varieties which are intentionally infected with *Pythium violae* or *Pythium sulcatum*
Date	**Milestone**
30/04/2017	2.1 Quality evaluation of the sampled plots described for milestone 2.1
30/06/2017	2.2 qPCR validation on the carrot collection described for milestone 2.1
Objective 3.	Selection of a final set of indicator genes that will be used for the test to determine the risk of the development of cavity spot
Date	**Milestone**
31/07/2017	3.1 A final set of carrot indicator genes
Objective 4.	Development of a user-friendly test format including decision criteria that will be beneficial to the growers
Date	**Milestone**
31/08/2017	4.1 A user friendly test format with decision criteria
Objective 5.	Continuous and active two-way communication between growers and researchers.
Date	**Milestone**
31/10/2017	5.1 A final report

Materials and methods

Collecting samples from the commercial fields

NSure commissioned Strawson as well as Poskitt to collect carrot samples from 15 different fields in August and October 2016. Per field, 2 replicate samples (A&B) of 25 carrots each had to be collected randomly from the field. The collected samples were transported via Elsoms Seeds to NSure to perform the sampling.

After washing, a peel was collected from each carrot using a potato peeler. The 25 slices were collected in a plastic bag, frozen in dry ice and stored in a -80 °C freezer until molecular analysis. NSure performed an additional sampling by using the NSure sampling kit (Figure 1). With the help of this method, juice was extracted and applied onto a special sampling card that fixed the genetic material.

Figure 1. Sampling procedure using the NSure sampling kit. 25 carrots were washed and from each carrot a vertical slice of 10 cm was collected with a potato peeler. The slices were transferred in the juice centrifuge and the RNA extraction fluid (NSure) was added. The juice was transferred in one half of the extraction bag containing a sieve. The pipette was used to suck up some juice from the other half of the bag. Two droplets of juice were applied to the sampling card. The cards were left for a couple of hours until dry.

Trial set-up inoculation trial and sample collection

Bejo Seeds in cooperation with Elsoms Seeds conducted two field trials, one on the premises of Elsoms Seeds and the other on a commercial field (Table 3, field 2) in Nottinghamshire. In each field, 6 varieties (Norway, Nerac, Nipomo, Norfolk, Newark and Nairobi) were intentionally infected with *Pythium violae*, *Pythium sulcatum* or an untreated control treatment. For each treatment and variety, four repetitions were laid down in randomized design at each field (Appendix 1 & 2).

The inoculation was performed according to the protocol developed by Suffert (2007) by inoculating *Pythium violae* or *Pythium sulcatum* on barley seeds and subsequently working it into the ground shortly before sowing.

In order to study gene expression, samples were collected individually from 2 repetitions (Rep 1 and Rep 3) in August and October 2016. Per repetition, Elsoms Seeds collected 25 carrots randomly from the plot which NSure used for frozen sampling.

Results

Quality evaluation performed on commercial fields in Nottinghamshire and Yorkshire

In collaboration with Strawson, Poskitt and Elsoms Seeds, NSure received samples from the most prominent carrot cultivar in the UK 'Nairobi' in August and October 2016 from various fields in the UK (Tables 3 & 4). In Nottinghamshire, the sampling was conducted according to plan. Due to unfortunate circumstances in Yorkshire (samples were accidently thrown away by a Poskitt employee, but they were able to collect some samples from other fields) we could not obtain a complete sample-set. Only in case of 4 fields (16, 18, 20 and 21), 2 replicate samples were collected in August as well as in October (Table 4). Of each sample received, NSure collected a frozen sample as well as a sample prepared using the NSure sampling kit which is used for commercial tests.

Table 3. Sample collection and field evaluation on the occurrence of cavity spot of commercial fields in Nottinghamshire

Field	Frozen sampling		Sampling card		Quality evaluation 1		Quality evaluation 2		Quality evaluation 3	
	Aug 2016	Oct 2016	Aug 2016	Oct 2016	%	Date	%	Date	%	Date
1	A&B	A&B	A&B	A&B	5	2-10-16	19	6-12-16	-	Harvested
2	A&B	A&B	A&B	A&B	23	2-10-16	-	Harvested	-	Harvested
3	A&B	A&B	A&B	A&B	0	2-10-16	2	8-12-16	-	-
4	A&B	A&B	A&B	A&B	22	2-10-16	-	Harvested	-	Harvested
5	A&B	A&B	A&B	A&B	0	2-10-16	0	8-12-16	-	-
6	A&B	A&B	A&B	A&B	2	2-10-16	0	8-12-16	-	-
7	A&B	A&B	A&B	A&B	0	2-10-16	1	8-12-16	-	-
8	A&B	A&B	A&B	A&B	0	2-10-16	5	8-12-16	33	Harvested
9	A&B	A&B	A&B	A&B	1	2-10-16	4	8-12-16	-	-
10	A&B	A&B	A&B	A&B	0	2-10-16	1	8-12-16	-	-
11	A&B	A&B	A&B	A&B	1	2-10-16	2	8-12-16	-	-
12	A&B	A&B	A&B	A&B	4	2-10-16	-	Harvested	-	Harvested
13	A&B	A&B	A&B	A&B	0	2-10-16	3	6-12-16	-	Harvested
14	A&B	A&B	A&B	A&B	0	16-10-16	3	8-12-16	-	-
15*	A&B	A&B	A&B	A&B	0	16-10-16	8	8-12-16	-	Harvested

* Pack-house intakes at harvest ranged from 0-21% cavity spot by weight

Strawson and Poskitt evaluated the fields for the occurrence of cavity spot by assessing 100 carrots randomly collected from the field. The cavity spot occurrence ranged from 0 to 33% (Tables 3 & 4). The cavity spot occurrence was lower in comparison to previous year. Strawson and Poskitt also checked the fields for the presence of other diseases and these observations are listed in Appendix 3 & 4. Scab was observed in various fields in Nottinghamshire, but also violet root rot, crown rot, nematode infestation and necrotic lesions due to viruses. In Yorkshire, mainly nematode infestations were observed.

Table 4. Sample collection and field evaluation on the occurrence of cavity spot of commercial fields in Yorkshire

Field	Frozen sampling		Sampling card		Quality evaluation 1		Quality evaluation 2		Quality evaluation 3	
	Aug 2016	Oct 2016	Aug 2016	Oct 2016	%	Date	%	Date	%	Date
16	A&B	A&B	A&B	A&B	0	13-09-16	0	03-01-17	0	13-02-17
17	A&B	-	A&B	-	0	13-09-16	0	03-01-17	0	13-02-17
18	A&B	A&B	A&B	A&B	0	13-09-16	0	03-01-17	1.42	13-02-17
19	A&B	-	A&B	-	0	13-09-16	0	04-01-17	14	Harvested
20	A&B	A&B	A&B	A&B	0	13-09-16	0	04-01-17	0	15-02-17
21	A&B	A&B	A&B	A&B	0	13-09-16	0	04-01-17	0	15-02-17
22	A&B	-	A&B	-	0	14-09-16	0	05-01-17	4.14	16-02-17
23	A&D	-	A&D	-	0	14-09-16	2.4	05-01-17	0	16-02-17
24	A&D	-	A&D	-	0	14-09-16	0	06-01-17	0	Harvested
25	A&D	-	A&D	-	0	14-09-16	0	06-01-17	0	Harvested
26	A&B	-	A&B	-	0	15-09-16	8,8/0*	07-01-17	0	17-02-17
27	A&B	-	A&B	-	0	15-09-16	0	07-01-17	0	17-02-17
28	-	A&D	-	A&D	0	15-09-16	0	07-01-17	0	18-02-17
29	-	A&B	-	A&B	0	15-09-16	0	07-01-17	0	18-02-17
30	-	A&B	-	A&B	0	15-09-16	0	07-01-17	4/0*	18-02-17
31	-	A&B	-	A&B	0	15-09-16	0	08-01-17	4/4.04*	18-02-17

*Two assessments were performed

Validation of the potential cavity spot indicators on samples collected from commercial fields in Nottinghamshire

Within the first year of the project, a set of potential indicator genes for cavity spot were identified by RNA-Seq. In the second year, these specific genes were validated for their suitability to serve as indicator genes to predict the risk of cavity spot. The gene expression profiles were first checked on the frozen samples collected from Nottinghamshire via qPCR as this sample-set was complete (Table 3). Several potential indicator genes were discarded as they did not show a consistent pattern towards each other and did not correlate to cavity spot occurrence.

Eleven genes remained that showed a quite similar gene expression pattern and 5 of them were highly comparable (Figure 2). For instance, all 5 genes were low expressed (red colour) in sample 1a-aug_2016, while they were high expressed in sample 5a_aug_2016 (green colour). From each field, 4 samples were collected (2 replicates in August and 2 in October). For the majority of the fields, a mutual gene expression pattern was observed for samples originating from the same field. Exceptions are for example between the samples collected from field 9; three samples showed low expression of the genes (red), while in sample 9b-aug_2016 the genes were highly expressed (green). This indicates that this field was highly heterogenous.

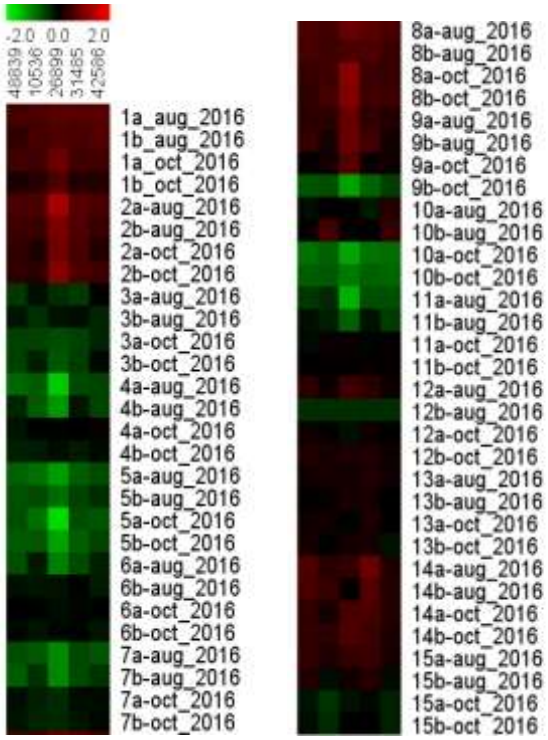

Figure 2. Heatmap representing colour-coded expression levels of 5 potential cavity spot indicator genes measured in all samples collected in Nottinghamshire in 2016 using qPCR. The colour bar on the left side demonstrates the log2 fold Ct difference between the gene of interest and stable housekeeping genes. Low expression of the gene is indicated in red, high expression in green. The numbers indicate field numbers, a/b the replicates and aug/oct samples collected in August/October.

A heatmap was also constructed from samples that originated from 15 fields collected in 2015 in Nottinghamshire (Appendix 5). For most fields, similar gene expression profiles were obtained between samples collected from the same field.

Based on the RNA-Seq data analysis performed in 2016, it seems that the 5 genes are lower expressed in cavity spot affected batches. Each gene has predictive power on its own, but a combination of genes is likely to give a more robust prediction. Therefore, an NSure Index value was calculated based on the sum of the gene expression values of the 5 genes (Table 5). Between the replicates samples, some variation in the NSure Index was encountered, demonstrating that there was some heterogeneity. This result shows that it is essential to collect more replicates in order to obtain a reliable impression of a field.

Table 5. NSure Index value determined in all samples collected in Nottinghamshire in 2016

Field	Samples August		Samples October		Quality evaluation		
	NSure Index		NSure Index		Occurrence of cavity spot (%)		
	A	B	A	B	October 2016	January 2017	February 2017
1	33	31	32	27	5	19	Harvested
2	36	32	30	32	23	Harvested	Harvested
3	18	19	15	16	0	2	-
4	12	15	21	19	22	Harvested	Harvested
5	12	14	12	13	0	0	-
6	15	20	21	20	0	0	-
7	13	14	19	19	0	1	-
8	33	31	32	32	0	5	33
9	29	29	25	13	1	4	-
10	22	25	11	12	0	1	-
11	14	17	22	22	1	2	-
12	26	17	22	25	1	4	Harvested
13	24	23	23	23	0	3	Harvested
14	31	29	29	29	0	3	-
15	27	23	19	20	0	8	Harvested

As the percentage of cavity spot changes over time in a field, it is impossible to create a test that can predict the actual percentage of occurrence of cavity spot. A high NSure Index value, only indicates that there is a high chance that cavity spot will occur in that particular field. Decision criteria should be defined to translate the combination of genes (NSure Index) in predictions. These predictions could be either ‘low risk’, ‘high risk’, or ‘indecisive’. Indecisive would be used for cases for which the outcome was in between high and low risk and no decision could be made. Although it was still too early to define the exact thresholds to mark to those classes, fields 1, 2, 8 and 14 would be considered as high risk fields based on the NSure Index. Farmers can work with an occurrence <15% of cavity spot relatively well, suggesting that it is most important to pick out the fields that show a high occurrence of cavity spot. Therefore, the cut-off for classifying a low risk batch and a high risk batch based on quality evaluation should be set at 15%. Quality evaluation demonstrated that fields 1, 2, 4 and 8 were high risk fields with a cavity spot occurrence >15%. Based on the NSure Index, fields 1, 2 and 8 would also be classified as high risk fields, but this would not be the case for field 4. According to the NSure Index value, field 14 was also assumed to be a high risk field. This did not correlate with the visual evaluation as in January a cavity spot occurrence of 3% was observed. Unfortunately, the field was not evaluated at a later stage. Based on the samples collected in August, field 9 would most probably also be classified as a risk field, but in October the NSure Index value dropped significantly in replicate B. The reason for this is

unclear, perhaps this was due to heterogeneity within the field. Although NSure was not able to select all the high risk fields based on the NSure Index, the genes were further validated in samples collected from Yorkshire and from the inoculation trial.

Validation of the potential cavity spot indicators on samples collected from commercial fields in Yorkshire

Sampling in Yorkshire was not performed according to plan, only in fields 16, 18, 20 and 21 were 2 replicate samples collected in August as well as in October (Table 4). The gene expression profiles of the 5 putative indicator genes were studied in the samples collected from the above-mentioned fields as well in samples collected from other fields with varying occurrences of cavity spot (Figure 3). The genes also showed a mutual gene expression pattern in the separate Yorkshire samples as was observed for the Nottingham samples.

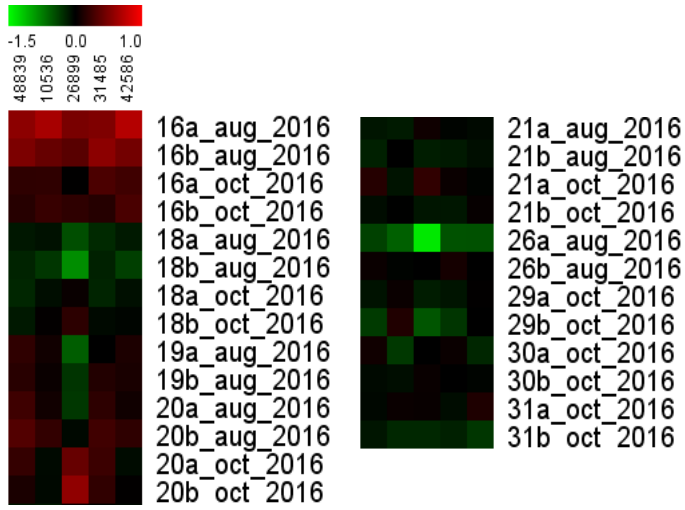

Figure 3. Heatmap representing colour-coded expression levels of 5 potential cavity spot indicator genes measured in various samples collected in Yorkshire in 2016 using qPCR. The colour bar on the left side demonstrates the log2 fold Ct difference between the gene of interest and stable housekeeping genes. Low expression of the gene is indicated in red, high expression in green. The numbers indicate field numbers, a/b the replicates and aug/oct samples collected in August/October.

All fields had a cavity spot occurrence <15%. Of all fields in Yorkshire, field 19 had the highest occurrence of cavity spot (Table 4). The NSure Index value was calculated (Table 6). Although an exact threshold was not set yet, the NSure Index values were roughly compared with the occurrence of cavity spot. Based on the NSure Index, it would be assumed that fields 16 and 20 were high risk fields. However, no cavity spot was encountered during quality evaluation.

Table 6. NSure Index value determined in all samples collected in Yorkshire in 2016

Field	Samples August		Samples October		Quality Evaluation		
	NSure Index		NSure Index		Occurrence of cavity spot (%)		
	A	B	A	B	September 2016	January 2017	February 2017
16	37	34	28	28	0	0	0
18	22	20	23	25	0	0	1.42
19	25	26	-	-	0	0	14
20	26	29	28	28	0	0	0
21	24	23	26	24	0	0	0
26	25	17	-	-	0	8.8/0*	0
29	-	-	24	22	0	0	0
30	-	-	24	24	0	0	4/0*
31	-	-	25	21	0	0	4/4.04*

*Two assessments were performed

Quality evaluation performed on the inoculation trial performed in Nottinghamshire

In cooperation with Bejo Seeds and Elsoms Seeds, two field trials were conducted in which 6 varieties (Norway, Nerac, Nipomo, Norfolk, Newark and Nairobi) were intentionally infected with *Pythium violae*, *Pythium sulcatum* or an untreated control treatment (Appendix 1 & 2).

One trial was conducted on a part of commercial field no. 2 in Nottinghamshire (Tables 3 & 5, Figure 2). The other trial was performed on the premises of Elsoms Seeds.

Bejo Seeds performed the quality evaluation on the samples originating from the inoculation trial executed in Nottinghamshire. In November 2016, prior to harvesting of the commercial field, approximately 20 carrots of each plot were sent to Bejo Seeds to be assessed. The occurrence (%) of cavity spot was determined as well as the severity.

Although most plots presented no to very little symptoms (Figure 4, left) of cavity spot, it could be observed that >20% of the carrots in the control treated plots were infected (Figure 4, right), demonstrating that there was already *Pythium* present in the soil. This finding corresponded with the commercially grown carrots in the same field. The grower encountered a cavity spot occurrence of 23% in field 2 (Table 3). As already *Pythium* was present in the soil, care should be taken with the outcome of the results.

A multiple comparison test (Tukey HSD test) was performed to investigate the significant differences between the three treatments independent of the variety (Figure 4). There was a significant difference observed between the control treated plots and the *Pythium sulcatum* treated plots (Figure 4). These results suggest that *Pythium sulcatum* was slightly more aggressive than *Pythium violae* or that the inoculum of *Pythium violae* was not that effective.

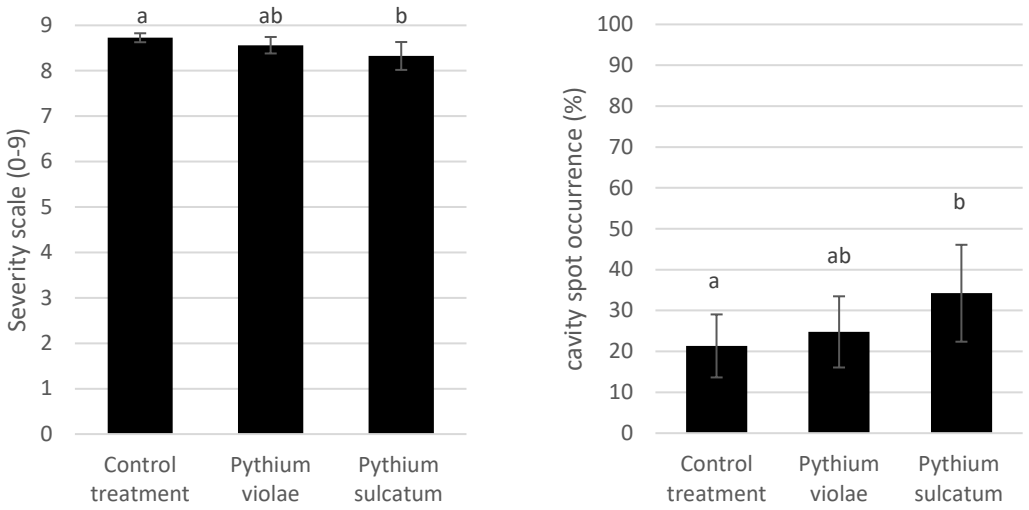

Figure 4. Cavity spot measurements conducted on the inoculation trial performed in Nottinghamshire. Left) Severity scale which ranges from 0 to 9; with 9 no symptoms observed and 0 very severely infected. Right) Cavity spot occurrence based on the total number (%) of infected carrots. Measurements were conducted in November 2016. Each bar represents the mean of all samples independent of the variety receiving that particular treatment. The error bar represents the standard error. Bars with different letters are significantly different (Tukey's HSD, $p<0.05$).

Next, the susceptibility of the six varieties towards the *Pythium* varieties was researched (Figure 5). Between the control treated varieties, no significant difference was encountered based on the severity as well as on the occurrence of cavity spot. Neither was this the case between the *Pythium violae* treatments. Between the varieties that were inoculated with *Pythium sulcatum* some significant differences could be observed, but this was not consistent between the two physiological measurements performed, except between Nerac and Nairobi. Based on this trial, it seems that Nairobi was more susceptible than Nerac. Although not significant, this trend was also observed between Nerac and Nairobi in the control- and *Pythium violae* treated plots. Based on the severity scale, Nipomo was also more susceptible than Nerac in the presence of *Pythium sulcatum*.

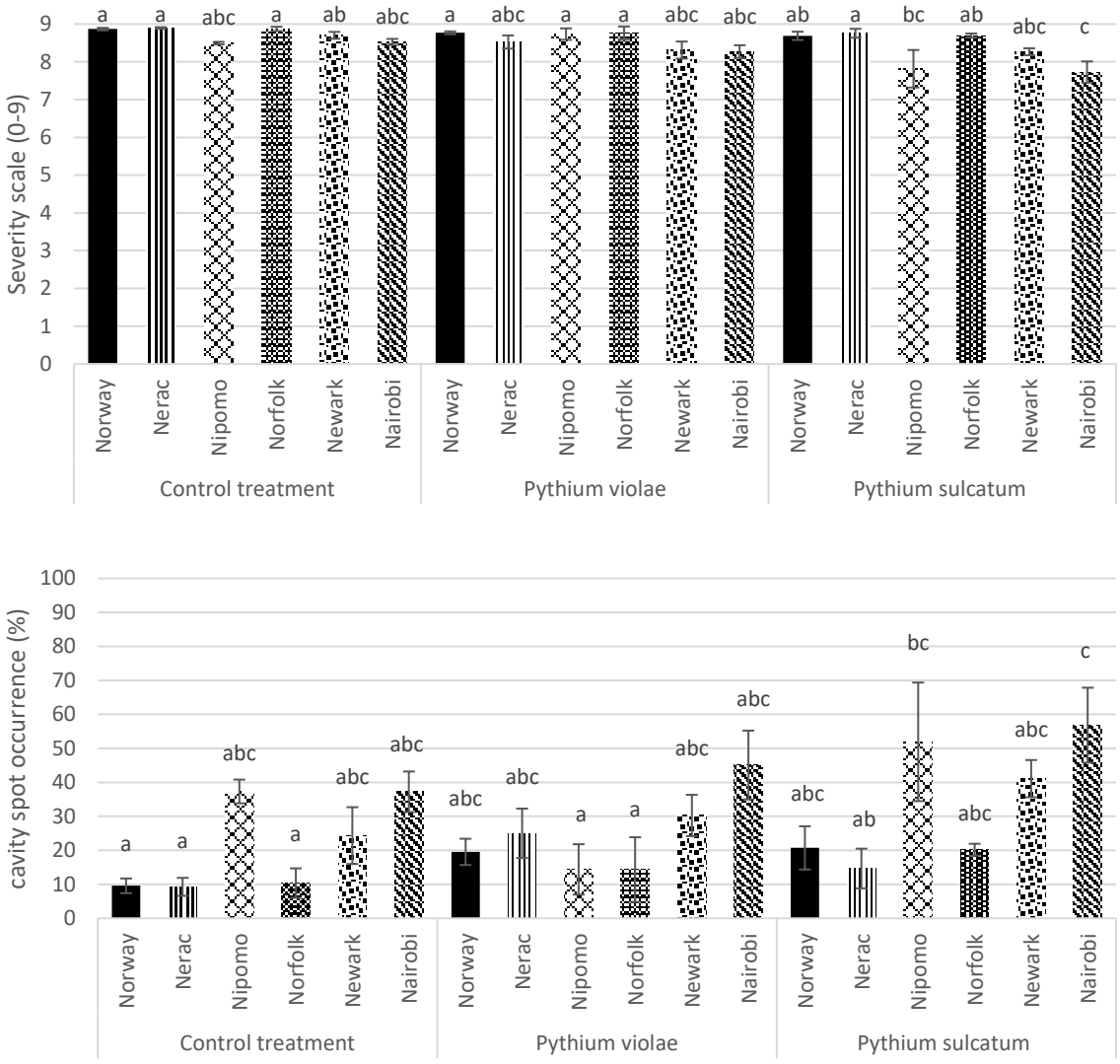

Figure 5. Cavity spot measurements conducted on the inoculation trial performed in Nottinghamshire. Top) Severity scale which ranges from 0 to 9; with 9 no symptoms observed and 0 very severely infected. Bottom) Cavity spot occurrence based on the total number (%) of infected carrots. Measurements were conducted in November 2016. Each bar represents the mean based on four repetitions. The error bar represents the standard error. Bars with different letters are significantly different (Tukey's HSD, $p<0.05$).

Quality evaluation performed on the inoculation trial performed on the premises of Elsoms Seeds

Elsoms Seeds evaluated the inoculation trial performed at their site. Assuming that later harvesting would give a better infestation, it was decided to postpone the quality evaluation to April 2016 as the level of infestation was very low in November. In April, 20 carrots of each plot were assessed on the number of lesions per root (Figure 6). In addition, the percentage of infected carrots was calculated (Figure 6).

During evaluation, a lot of damage was observed by violet root rot and carrot root fly. This was so severe that in most cases it was impossible to score the carrots for cavity spot as the violet root rot and carrot fly symptoms masked the underlying cavity spot lesions. Therefore, care should be taken with the interpretation of the results.

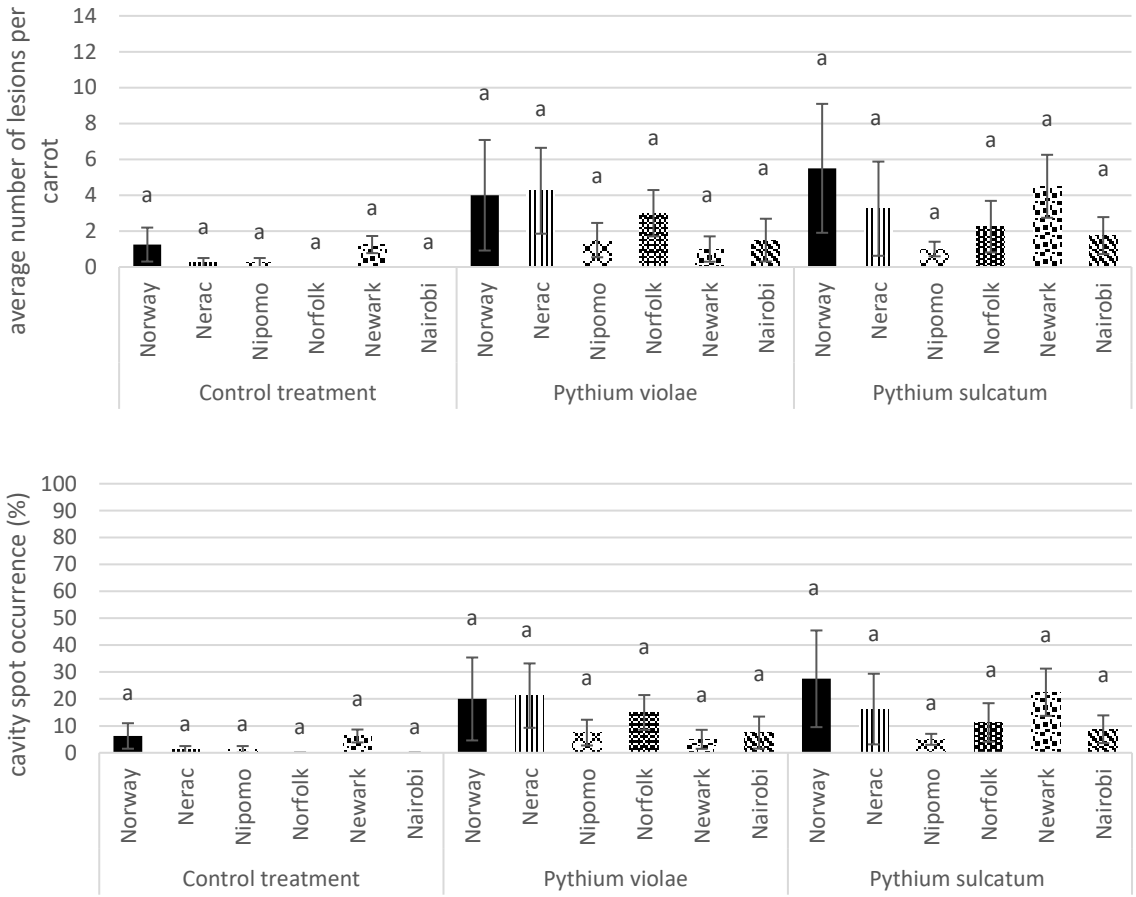

Figure 6. Cavity spot measurements conducted on the inoculation trial performed on the premises of Elsoms Seeds. Top) Average number of lesions. Bottom) Cavity spot occurrence (%). Measurements were conducted in April 2016. Each bar represents the mean based on four repetitions. The error bar represents the standard error. Bars with different letters are significantly different (Tukey's HSD, $p<0.05$).

Due to a huge variation in the number of lesions and occurrence of cavity spot between the replicates, no significant differences between the varieties were observed.

A significant difference was observed between the control treated plots and the *Pythium sulcatum* treated plots based on the cavity spot occurrence (Figure 7). This was also observed in the trial conducted in Nottinghamshire (Figure 4).

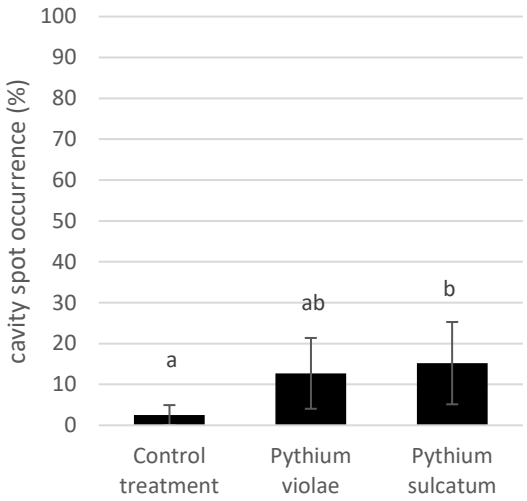

Figure 7. Cavity spot measurements conducted on the inoculation trial performed on the premises of Elsoms Seeds. Cavity spot occurrence based on the total number (%) of infected carrots. Measurements were conducted in November 2016. Each bar represents the mean of all samples independent of the variety receiving that particular treatment. The error bar represents the standard error. Bars with different letters are significantly different (Tukey's HSD, $p<0.05$).

Validation of the potential cavity spot indicators on samples collected from the inoculation trial in Nottinghamshire

In Figure 8, the gene expression profiles are shown of the 5 potential indicator genes measured in all samples collected during the inoculation trial in Nottinghamshire. Similar, to samples collected from the commercial fields (Figures 2 and 3), it can be observed that most genes showed a mutual gene expression pattern in each separate sample. Strikingly, in most cases the 5 genes were lower expressed in the samples collected in August in comparison to October regardless of the variety and this was also observed when looking at the NSure Index value (Table 7). This difference was not observed in the samples originating from the commercial fields.

No correlation was observed for the NSure Index values generated in August and/or October in comparison with the severity scale and occurrence. Based on the NSure Index values

generated in August, all analysed plots would be classified as high-risk fields, while in October most plots would be labelled as low risk fields.

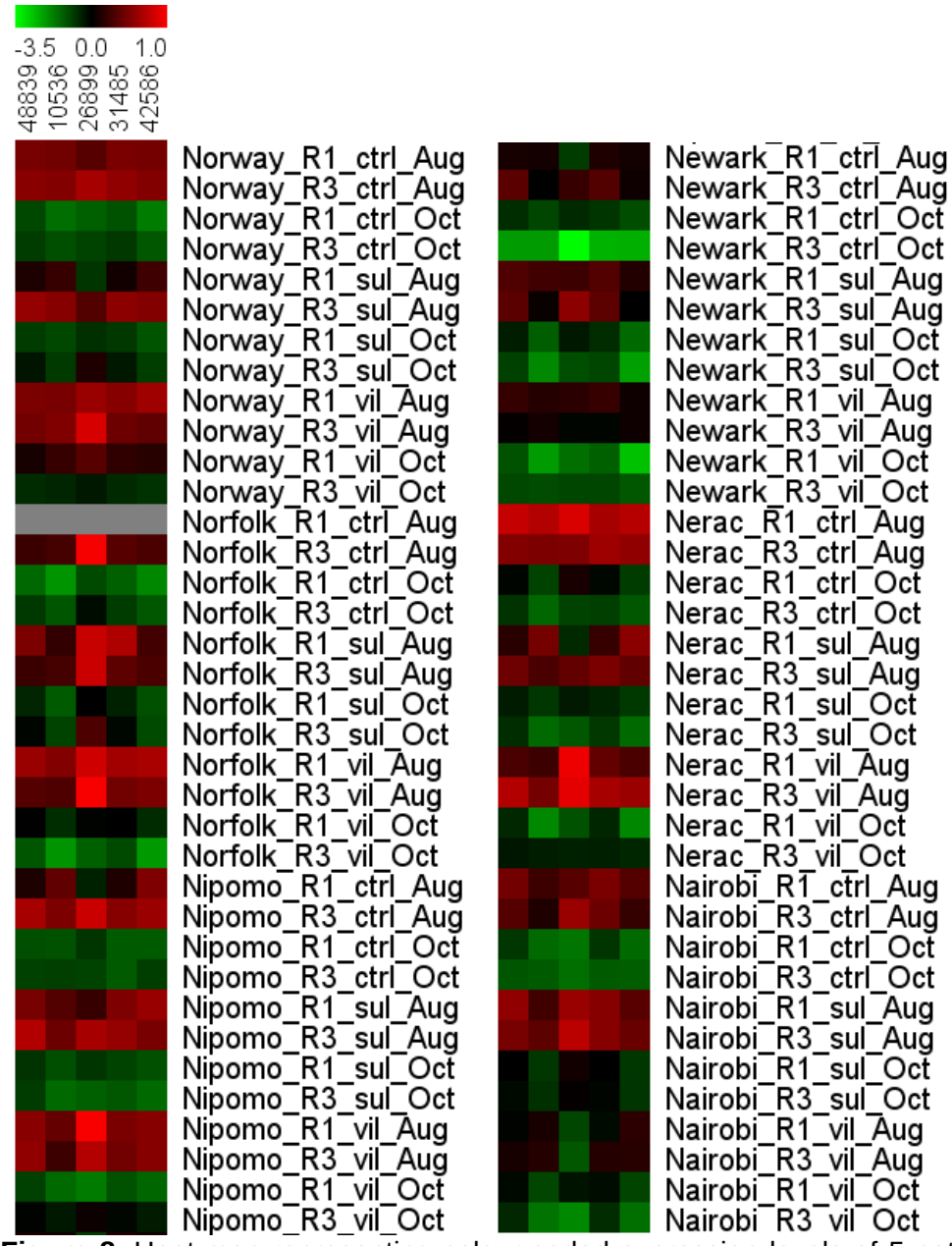

Figure 8. Heat map representing colour-coded expression levels of 5 potential cavity spot indicator genes measured in all samples collected during the inoculation trial performed in Nottinghamshire using qPCR. The colour bar on the left side demonstrates the log2 fold Ct difference between the gene of interest and stable housekeeping genes. Low expression of the gene is indicated in red, high expression in green. R1/R3 indicates the replicate number, ctrl for control, sul for *Pythium sulcatum* vil for *Pythium violae* and aug/oct samples collected in August/October.

Table 7. NSure Index value determined in all samples collected from the inoculation trial executed in Nottinghamshire

Variety	Treatment	Repetition	NSure Index		Severity scale (0-9)	Occurrence (%)
			August	October		
Norway	**control**	1	41	12	8.9	15.0
Norway	**control**	3	45	15	8.9	8.7
Norway	***P. sulcatum***	1	31	16	8.5	33.3
Norway	***P. sulcatum***	3	43	23	8,5	30.0
Norway	***P. violae***	1	44	35	8.8	22.7
Norway	***P. violae***	3	44	21	8,9	9.1
Nerac	**control**	1	51	24	8.9	15.0
Nerac	**control**	3	44	15	8.9	12.5
Nerac	***P. sulcatum***	1	36	21	8.8	20.0
Nerac	***P. sulcatum***	3	40	14	9.0	4.8
Nerac	***P. violae***	1	41	14	8.8	15.8
Nerac	***P. violae***	3	48	22	8.1	33.3
Nipomo	**control**	1	35	14	8.4	45.5
Nipomo	**control**	3	46	16	8.6	28.6
Nipomo	***P. sulcatum***	1	41	16	8.4	31.8
Nipomo	***P. sulcatum***	3	45	13	7.6	81.0
Nipomo	***P. violae***	1	46	12	8.3	35.0
Nipomo	***P. violae***	3	43	27	9.0	0.0
Norfolk	**control**	1	-	10	8.9	7.1
Norfolk	**control**	3	41	17	9.0	0.0
Norfolk	***P. sulcatum***	1	42	19	8.8	20.0
Norfolk	***P. sulcatum***	3	40	24	8.7	19.0
Norfolk	***P. violae***	1	48	25	9.0	4.8
Norfolk	***P. violae***	3	43	11	9.0	5.0
Newark	**control**	1	30	17	8.6	29.4
Newark	**control**	3	34	6	8,7	16.7
Newark	***P. sulcatum***	1	36	18	8.4	38.9
Newark	***P. sulcatum***	3	36	12	8.0	57.1
Newark	***P. violae***	1	34	9	8.5	26.3
Newark	***P. violae***	3	30	15	8.6	18.8
Nairobi	**control**	1	39	13	8.4	31.3
Nairobi	**control**	3	38	12	8,7	27.8
Nairobi	***P. sulcatum***	1	42	25	7.7	73.7
Nairobi	***P. sulcatum***	3	43	24	7.1	75.0
Nairobi	***P. violae***	1	28	22	7.8	61.1
Nairobi	***P. violae***	3	30	14	8.2	63.6

Validation of the potential cavity spot indicators on samples collected from the inoculation trial on the premises of Elsoms Seeds

The inoculation trial executed at the premises of Elsoms Seeds suffered from a high damage due to violet root rot and carrot root fly. In many cases, it was impossible to evaluate the carrots on cavity spot and therefore the quality evaluation results cannot be trusted. As the quality evaluation results were not reliable, samples could not be used to correlate it to the gene expression profiles.

Although this comparison could not be performed, it was decided to analyse the samples from the plots growing Nairobi and Nerac to investigate whether there was a difference in gene expression between the samples collected in August and October. The heatmap and the corresponding NSure Index values are shown in Figure 9 and Table 8, respectively. The mutual gene expression profiles that were observed between the 5 genes in the other sample-sets was less pronounced in the samples collected from this trial. Furthermore, the clear difference that was observed between the 'August' and 'October' samples that were collected from the inoculation trial executed in Nottinghamshire was less pronounced within this trial (this difference was only observed for a few plots). No correlation was observed for the NSure Index values generated in August and/or October in comparison with the number of lesions and occurrence.

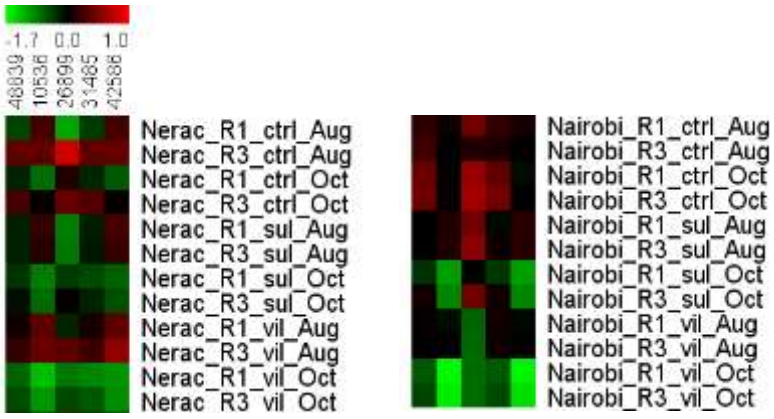

Figure 9. Heat map representing colour-coded expression levels of 5 potential cavity spot indicator genes measured in all samples from Nerac and Nairobi collected during the inoculation trial performed on the premises of Elsoms Seeds using qPCR. The colour bar on the left side demonstrates the log2 fold Ct difference between the gene of interest and stable housekeeping genes. Low expression of the gene is indicated in red, high expression in green. R1/R3 stands for the replicate number, ctrl for control, sul for *Pythium sulcatum* and vil for *Pythium violae*.

Table 8. NSure Index value determined in all samples collected from the inoculation trial executed on the premises of Elsoms Seeds

			NSure Index			
Variety	**Treatment**	**Repetition**	**August**	**October**	**Average no. of lesions**	**Occurrence (%)**
Nerac	**control**	**1**	32	27	0	0
Nerac	**control**	**3**	48	40	0.05	5
Nerac	***P. sulcatum***	**1**	34	24	0	0
Nerac	***P. sulcatum***	**3**	33	28	1	55
Nerac	***P. violae***	**1**	40	18	0.3	20
Nerac	***P. violae***	**3**	45	21	0.95	55
Nairobi	**control**	**1**	39	41	0	0
Nairobi	**control**	**3**	37	43	0	0
Nairobi	***P. sulcatum***	**1**	38	23	0	0
Nairobi	***P. sulcatum***	**3**	39	30	0.25	15
Nairobi	***P. violae***	**1**	33	17	0.85	25
Nairobi	***P. violae***	**3**	33	18	0	0

Optimisation sampling procedure using the NSure sampling kit

Alongside frozen samples, juice samples were collected using the specific sampling card. From a sampling card, only a limited amount of genetic material can be obtained. This amount is sufficient once a test is developed, but not for research purposes. It was essential to know whether the 5 potential indicator genes showed similar gene expression patterns when genetic material was isolated from the cards. The gene expression values obtained from frozen samples collected from the commercial fields in Nottinghamshire were compared to the corresponding sampling card samples (Table 3). Regression analysis on the 5 genes are depicted in Figure 10. The R-squared values obtained for these 5 genes were high (this should be near 1) considering that different sampling methods and RNA isolation protocols were used. The results show that if those genes would comprise the test, that growers easily can take a sample using the NSure sampling kit instead of frozen samples.

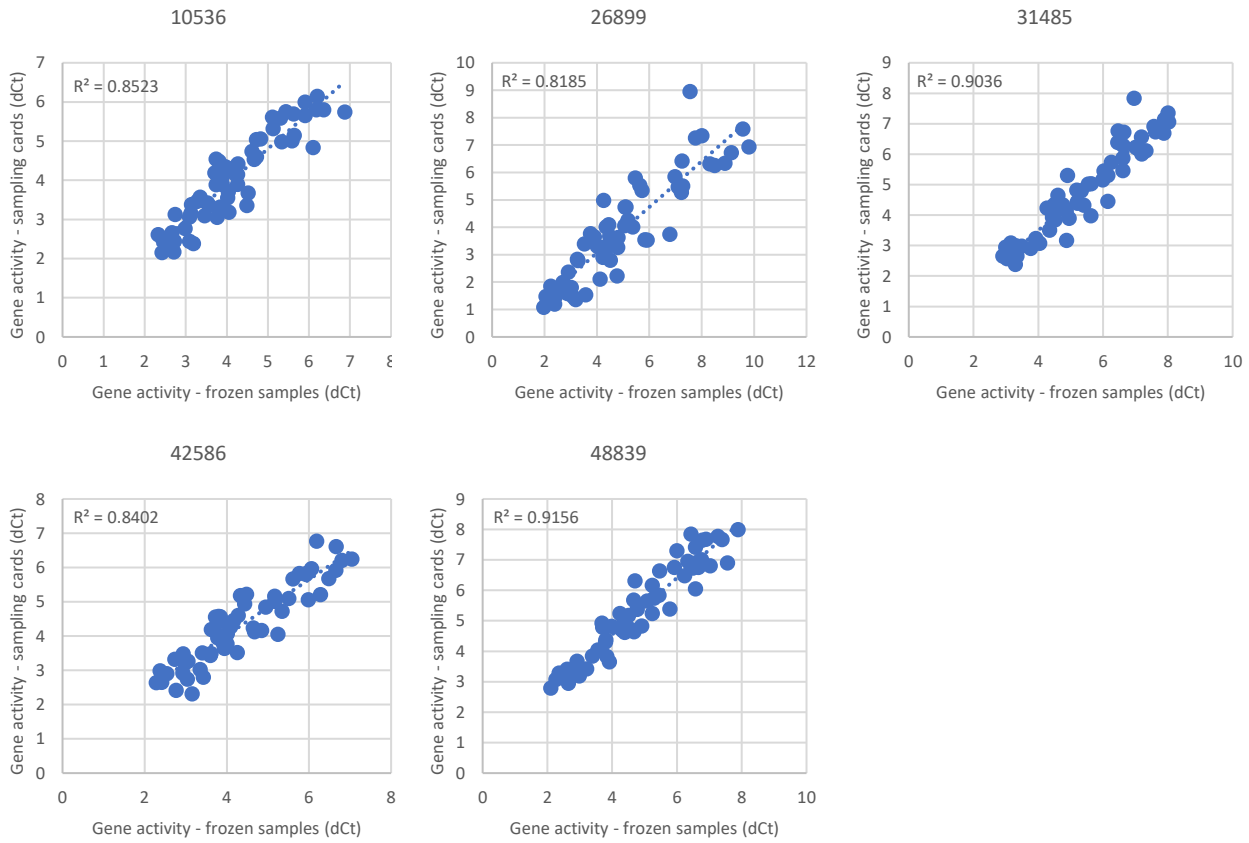

Figure 10. Linear regression between the gene expression results of the sampling card samples to the corresponding frozen samples obtained from the commercial fields in Nottinghamshire (Table 2). The level of gene expression was given as the dCt, which is the Ct difference between the gene of interest and stable housekeeping genes.

For the StoreNSure black spot test, NSure developed a sampling procedure using the NSure sampling kit. Using this method, juice is extracted from carrot peels with help of a juice centrifuge which is then applied on the sampling card. Although this procedure works very well as can be observed in Figure 10, it would be a great advantage if the juice centrifuge could be omitted. This is because, not every juice centrifuge is suitable to use, the customer needs to buy one and cleaning is needed.

To circumvent the juice centrifuge step, NSure tested several other ways to extract juice out of carrot peels. After performing multiple extractions tested by different people, it turned out that crushing the peels with a hammer led to similar amounts of RNA and gene expression results. The optimized sampling procedure for carrot peels using the NSure sampling kit is depicted in Figure 11.

Figure 11. Sampling procedure using the NSure sampling kit. Select 25 representative carrots. Wash the carrots and collect from each carrot a vertical slice of 10 cm with a potato peeler. Combine all slices in a single compartment of the extraction bag and add the RNA extraction fluid (NSure). Close the bag and mash the pieces with a hammer to a fine consistency. Mash max. 1 min. Collect some juice from the other compartment using the pipette. Apply 2 drops of juice inside the circle on the sampling card. Air dry the card for at least 2 hours. The card must be completely dry. Insert dried card into the grip seal bag with drying agent.

Discussion

The intended time projected to develop a molecular test to detect cavity spot at an early stage using carrot specific genes was expected to require two years of research.

In the first year of the project, a longlist of potential indicator genes for cavity spot was identified using RNA-Seq. The most promising indicator genes were scrutinized by testing their predicting power on a large selection of the samples collected in 2015 by using qPCR. Several potential indicator genes survived this testing phase, although it should be noted that the correlation between the gene expression profiles to the occurrence of cavity spot was not 100%.

The predictive power of the potential indicator genes, identified in year 1, were validated in various sample sets in year 2. Validation of the potential indicator genes was hampered by a variety of issues (incomplete sample set, limited amount of quality assessments, presence of other pathogens that masked the cavity spot lesions; and variability in the occurrence of cavity spot within the field) which are discussed below.

A complete sample-set was obtained from the commercial fields in Nottinghamshire, but due to harvesting only field 8 was evaluated three times. That multiple evaluations are important to obtain a good impression of the field quality was clearly shown for field 8. In September, the occurrence of cavity spot was 0%, in January 5% and in February it raised to 33%. A complete sample set from the commercial fields in Yorkshire was not obtained and this hampered the validation. Four fields were sampled the proper way; 2 replicate samples in August and 2 replicate samples in October. Quality evaluation was determined three times. The occurrence of cavity spot in the commercial fields was lower in comparison to 2015. This was positive for the growers, but this was disadvantageous for the validation of the potential indicator genes.

In case of the inoculation trials, several complications were encountered. In both trials, the severity of cavity spot was low. Based on the control treatment, it could be deduced that there was already *Pythium* present in the fields (especially in Nottinghamshire). In both trials, no significant differences could be observed between the control treated plots and the *Pythium violae* treated plots irrespective of the tested varieties, but there was a significant difference between the control treated plots and the *Pythium sulcatum* treated plots based on the number of infected carrots. These results imply that *Pythium sulcatum* was slightly more aggressive than *Pythium violae* or that the inoculum of *Pythium violae* was not that effective. Although the severity of cavity spot was low, the number of infected carrots was high in various plots. The occurrence of cavity spot was highly variable between certain replicates and this was most pronounced in the inoculation trial performed at Elsoms Seeds. Both trials

had to cope too with the presence of other pathogens. The inoculation trial executed at Elsoms Seeds suffered from a high damage due to violet root rot and carrot root fly that in all probability have masked the cavity spot lesions leading to results that cannot be relied upon. The inoculation trials were only evaluated once on cavity spot. Regarding the trial conducted at Elsoms Seeds, no significant differences were observed in susceptibility between the tested varieties towards *Pythium sulcatum* and *Pythium violae*. The huge variability between the replicates and the presence of other diseases that have masked the cavity spot lesions might have contributed to the fact that no significant differences were observed between the varieties. In the presence of *Pythium sulcatum*, a significant difference was observed between Nerac and Nairobi in the Nottinghamshire inoculation trial. Although not significant, this trend was also observed between Nerac and Nairobi in the control and *Pythium violae* treated plots. Nairobi seems to be more susceptible variety than Nerac. Based on the severity scale, Nipomo was also more susceptible in the presence of *Pythium sulcatum* than Nerac. Within this trial, there appears to be a trend that Nerac, Norfolk, Norway have the same tolerance level against cavity spot whereas Nipomo, Newark and Nairobi seem to be the more susceptible ones. These results were similar to those obtained in a cavity spot field trial performed by Bejo Seeds in the Netherlands in 2015 (personal communication).

The expression of the potential indicator genes, identified in year 1, were initially checked in the frozen samples collected from the commercial fields in Nottinghamshire in 2016. Several genes were discarded based on their gene expression pattern as they did not correlate with the cavity spot occurrence. Eleven genes remained, as they showed a quite similar gene expression pattern towards each other and of this set of genes, 5 were highly comparable. Based on the RNA-Seq study performed previous year, the genes were lower expressed in the high risk batches in comparison to the low risk batches. For the majority of the fields, the gene expression profiles as well as the NSure index values were quite comparable between samples collected from the same field irrespective of the time when collected. However, for certain fields, variation was observed between the replicates or time points, demonstrating that there may have been some heterogeneity within the field. The results show that it is essential to collect at least 2 replicates to obtain an impression of a field.

General functions were assigned to the 11 genes by comparing the genes with academic databases. Five genes could be associated with ethylene signalling, 3 genes that respond to (a)biotic stresses, 1 gene that facilitates gene expression and 2 genes with an unknown function. It was quite striking that 5 out 11 genes are involved in ethylene signalling. The plant hormone ethylene regulates many developmental processes such as seed germination, growth of roots and shoots, fruit ripening and senescence, but is also involved in plant pathogen interactions. Root invasion by *Pythium spp.* is characterized by degradation of host

cell walls and plants may respond actively to a Pythium invasion by thickening and lignification of the wall. Ethylene has shown to alter lignification, cell wall synthesis and cell wall composition (Geraats *et al.*, 2002). Furthermore, ethylene insensitive tobacco and *Arabidopsis* mutants showed increased susceptibility to *Pythium spp.* (Geraats *et al.*, 2002).

In case of the commercial fields in Nottinghamshire, NSure was not able to pick out all the high-risk fields. Although the correlation was not conclusive, it still looked promising especially regarding the predicted function of the genes. The set of genes were further validated in samples collected from commercial fields in Yorkshire and during the inoculation trials. For most samples measured, similar gene activity profiles were observed between the genes. Looking at field level, some clear differences between the sample-sets could be observed. The gene expression profiles as well as the NSure index values were quite comparable between the samples collected from the same commercial fields in Yorkshire. However, in case of the inoculation trial performed in a commercial field in Nottinghamshire (in the other trial it was less pronounced) huge differences were observed in gene expression between most of the samples collected in August in comparison to the samples collected in October. This inoculation trial was organized in a part of commercial field 2 (Table 3) in which a cavity spot occurrence of 23% was observed. From the commercially grown carrots in field 2, also samples were collected in August and October. In case of those samples, we did not encounter a difference in gene expression in time. Neither did we for the samples collected from other commercial fields in 2015 and 2016. Currently, it is unclear what caused this huge difference in gene expression in the carrots collected from this inoculation trial.

No correlation was observed between the NSure Index values and the occurrence of cavity spot which leads us to question whether these genes are truly cavity spot indicators. It could be that the genes are regulated by something else, but at the moment it is unclear what it could be. Gene expression does not change over time for all fields, except for the inoculation trial. So, it is unlikely that the expression of the selected genes changed due to maturation or for example a declining soil temperature. Furthermore, it could also not be related with the presence of other pathogens. The RNA-Seq study was re-evaluated again to find some new potential genes. Some candidate genes were found, but they did not pass the qPCR validation.

We have been able to optimize the “grower friendly” sampling protocol by creating a new sampling protocol that does not need a juice centrifuge.

The whole project made clear that carrots suffer from a diversity of pathogens. This hampered test development as it can be imagined that other pathogens may trigger the same genes as *Pythium,* making it difficult to create a test that is specific for cavity spot. It was expected that

the inoculation trial would lead to a complete sample-set of non-infected samples (control treatment) and *Pythium sulcatum/Pythium violae* infected samples that could reinforce the validation, but unfortunately the trials had to cope with other pathogens and cavity spot also occurred in the control treated plots.

Future work

This project made clear that it will be difficult to find specific genes for cavity spot. It could be that there are no specific genes that are solely altered upon a *Pythium* infection. For instance, the black spot test is a test compromising plant defence genes that are triggered by all black spot related fungi and *Phytophthora*. On the other hand, the sample-set variability seemed to hamper the identification of specific genes. A different approach could possibly lead to the identification of specific indicator genes. First, an inoculation trial with *Pythium sulcatum/Pythium violae* should be set up under tight controlled conditions to assure the absence of pathogens in order to find true cavity spot indicators. Samples should be collected from the control inoculated plots at several timepoints and the occurrence of cavity spot should be monitored thoroughly. RNA-Seq should be performed to identify all the genes that are altered upon a *Pythium* infection and this information can also be used to unravel the underlying defense mechanisms which are altered upon a *Pythium* infection in carrot. Next, the expression profiles of potential indicator genes should be monitored in the field for several years to select the specific genes and discard the generic genes that also respond to another pathogen infection or certain condition. As well as thoroughly monitoring the occurrence of cavity spot, it would also be valuable to monitor the presence of *Pythium* in the soil and on the carrot itself. This would lead to a better understanding of the disease and possibly development of a molecular test.

Conclusions

The time projected to develop a molecular test to detect cavity spot at an early stage using carrot specific genes appeared to be ambitious. This project shows that this particular disease is very unpredictable and that is difficult to find genuine patterns. In addition, the difficulty to obtain large reliable sample sets with sufficient quality evaluation moments complicates the search and validation of specific indicators.

A different approach could possibly lead to specific indicator genes. First, an inoculation trial should be organized under tight controlled conditions to identify potential cavity spot indicator genes. After identification, the potential indicator genes should be tightly monitored in the field over in combination with other measures to find genuine patterns and reliable genes.

Knowledge and Technology Transfer

The technology transfer activities carried out that relate to this project:

Activity	To	Date
Summary of the progress	BCGA	22-03-2016
Oral presentation	Poskitt, Strawson, Elsoms Seeds, AHDB	28-06-2016
Newsletter NSure	People subscribed to our newsletter. It can also be viewed on our website	06-09-2016
Summary of the progress	BCGA	13-10-2016
Annual report	AHDB	25-11-2016
Summary of the progress	AHDB	10-04-2017
Summary of the progress	BCGA	07-06-2017
Oral presentation	British Carrot and Onion Conference	14-11-2017
Oral presentation	Poskitt, Strawson, Elsoms Seeds, AHDB	15-11-2017

Glossary

Down-regulated	describes a gene which has been observed to have lower expression in one sample compared to another
Gene expression	the process by which a gene is turned on to produce the specific biological molecule (RNA) encoded by that gene. Lowering gene expression leads to less RNA of this particular gene, while induction leads to more RNA (and thus a higher gene expression)
Housekeeping gene	a gene that is expressed at a relatively constant level across many known conditions
RNA	a nucleic acid that is the primary product of gene expression
RNA sequencing (RNA-Seq)	method to reveal the presence and quantity of RNA (from the active genes) in a sample at a given moment in time
Quantitative RT PCR (qPCR)	method to specific quantify the RNA you are interested in
Up-regulated	describes a gene which has been observed to have higher expression in one sample compared to another

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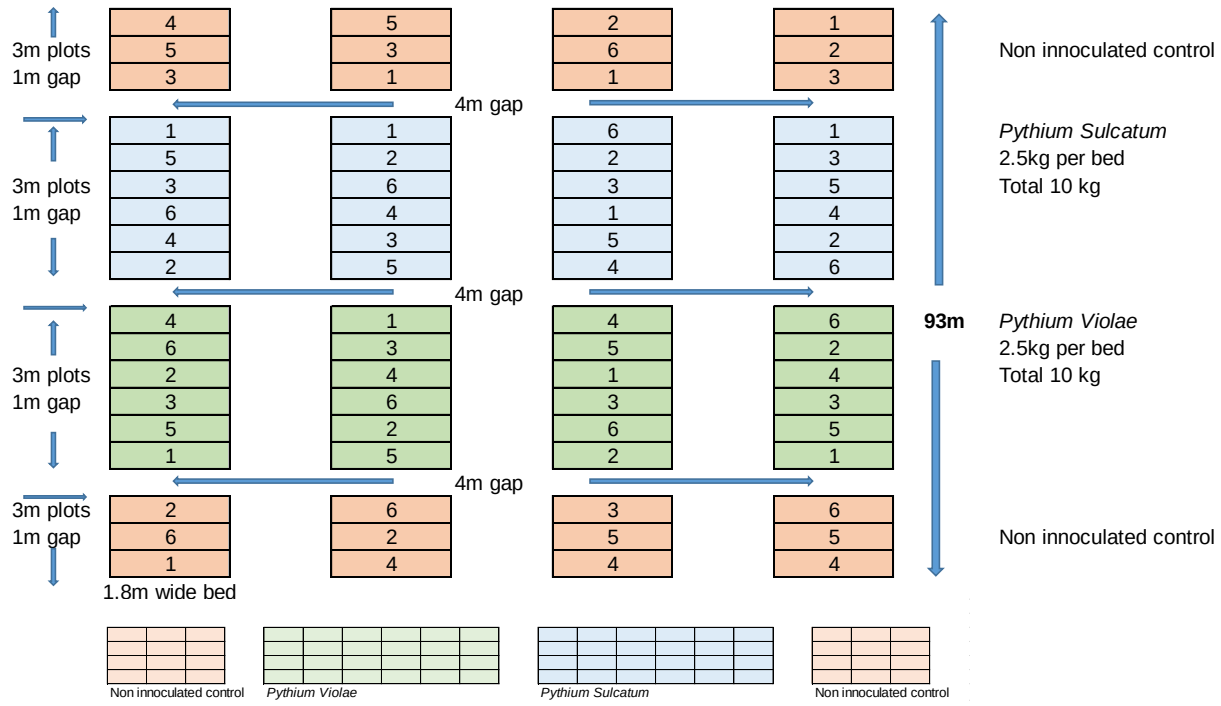
Appendix 1

Trial at Elsoms Seeds

Density 80 seeds / linear meter
4 x triple rows
4 rows per variety 4 repetitions

1 Norway 4 Norfolk
2 Nerac 5 Newark
3 Nipomo 6 Nairobi

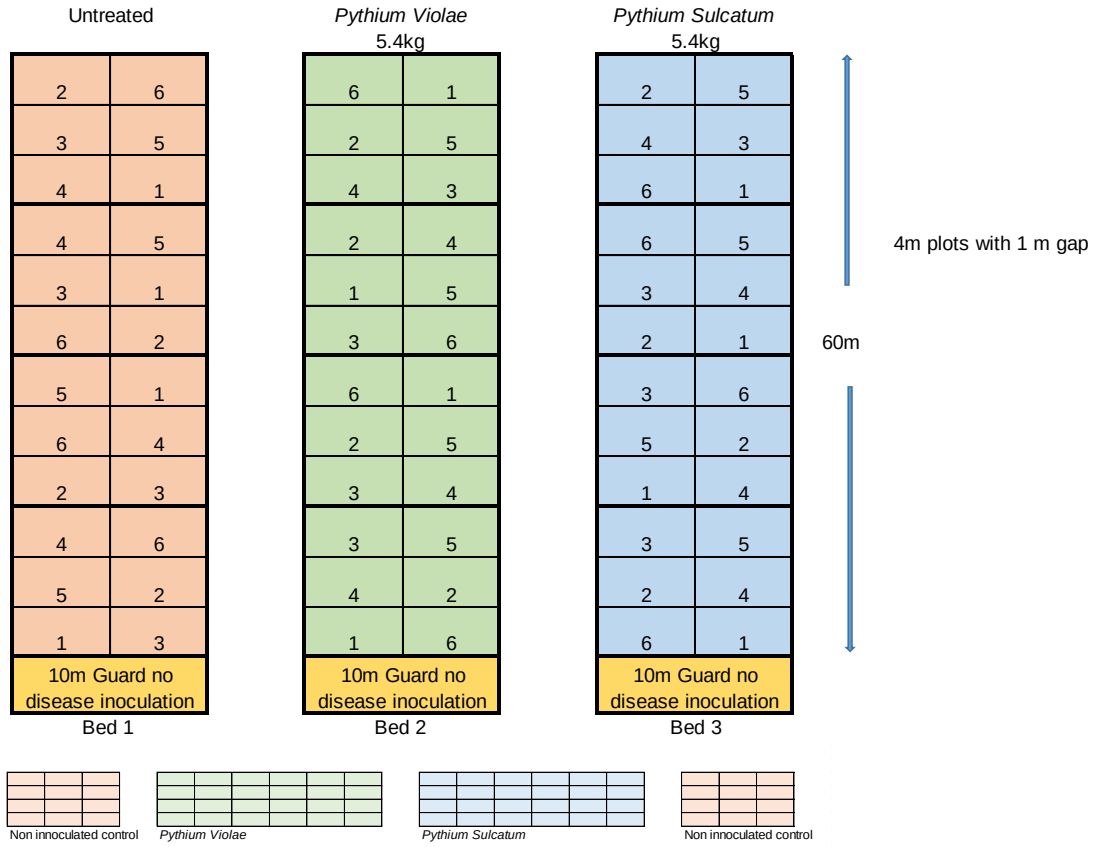


Appendix 2

Trial in Nottinghamshire

Density 80 seeds / linear meter
4 x triple rows 320 seeds / triple row
2 rows per variety 4 repetitions

1 Norway 4 Norfolk
2 Nerac 5 Newark
3 Nipomo 6 Nairobi



Appendix 3

Evaluation of fields in Nottinghamshire

Field	Quality evaluation 1		Quality evaluation 2	
	Date	% of other diseases	Date	% of other diseases
1	2-10-16	scab (1), virus (6), nematode (7)	6-12-16	virus (3), scab (1)
2	2-10-16	scab (10), nematode (6)	harvested	-
3	2-10-16	scab (9), nematode (1), virus (1), crown rot (1)	8-12-16	virus (2), nematode (2)
4	2-10-16	crown rot (4), virus (4), nematode (2)	harvested	-
5	2-10-16	virus (2), scab (1), nematode (1)	8-12-16	scab (12), virus (3), crown rot (2), nematode (2)
6	2-10-16	virus (2), crown rot (1), nematode (7)	8-12-16	pest damage (2), scab (3), nematode (5), virus (3), crown rot (2)
7	2-10-16	virus (5)	8-12-16	virus (18), nematode (1)
8	2-10-16	nematode (6), crown rot (1), scab (3), virus (1)	8-12-16	virus (1), nematode (4)
9	2-10-16	nematode (2), scab (2), virus (3)	8-12-16	-
10	2-10-16	virus (5), crown rot (1), nematode (5)	8-12-16	crown rot (3), virus (3), scab (1), nematode (1), carrot fly (5)
11	2-10-16	nematode (3), scab (3), virus (4)	8-12-16	nematode (13), rot (1)
12	2-10-16	scab (11), crown rot (1), violet root rot (1)	harvested	-
13	2-10-16	scab (16), nematode (4), virus (3)	6-12-16	violet root rot (2), scab (1), virus (6), carrot fly (2)
14	16-10-16	violet root rot (6), scab/blemish/scar (3)	8-12-16	violet root rot (1), nematode (1)
15	16-10-16	scab (2)	8-12-16	nematode (3)

* At quality evaluation moment 3 carrots were not evaluated for other diseases except for cavity spot.

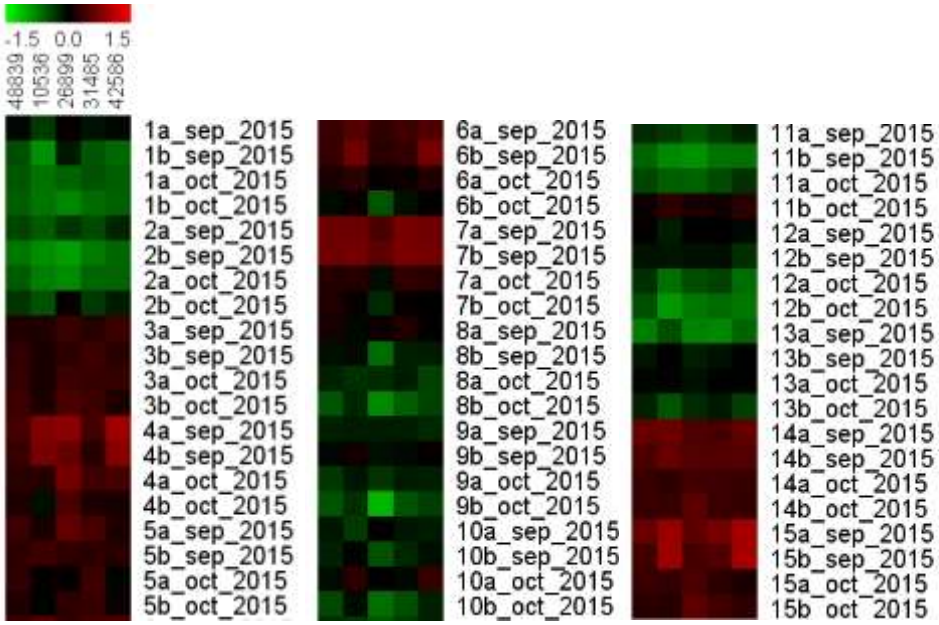
Appendix 4

Evaluation of fields in Yorkshire

Field	Quality evaluation 1		Quality evaluation 2		Quality evaluation 3	
	Date	% of other diseases	Date	% of other diseases	Date	% of other diseases
16	13-09-16	nematode (2/0)	03-01-17	nematode (4)	13-02-17	? (1)
17	13-09-16	0	03-01-17	0	13-02-17	? (1)
18	13-09-16	nematode (7/5)	03-01-17	nematode (16)	13-02-17	? (3)
19	13-09-16	nematode (3/0)	04-01-17	nematode (5)	harvested	-
20	13-09-16	nematode (5/3)	04-01-17	nematode (6)	15-02-17	
21	13-09-16	nematode (1/0)	04-01-17	nematode (3)	15-02-17	? (1)
22	14-09-16	0	05-01-17	0	16-02-17	? (1)
23	14-09-16	virus (7)	05-01-17	nematode (15)	16-02-17	? (4) ? (4)
24	14-09-16	nematode (4)	06-01-17	nematode (7)	harvested	-
25	14-09-16	0	06-01-17	nematode (8)	harvested	-
26	15-09-16	0	07-01-17	nematode (1)	17-02-17	0
27	15-09-16	nematode (2)	07-01-17	0	17-02-17	crown rot (4) scab (4)
28	15-09-16	0	07-01-17	0	18-02-17	0
29	15-09-16	0	07-01-17	0	18-02-17	0
30	15-09-16	0	07-01-17	0	18-02-17	0
31	15-09-16	0	08-01-17	0	18-02-17	0

Appendix 5

Heat map representing colour-coded expression levels of 5 potential cavity spot indicator genes measured in all samples collected in 2015 in Nottinghamshire using qPCR. The colour bar on the left side demonstrates the log2 fold Ct difference between the gene of interest and stable housekeeping genes.



NSure Index value determined in all samples collected in Nottinghamshire in 2015

Field	Samples August NSure Index		Samples October NSure Index		Quality evaluation Occurrence of cavity spot (%)	
	A	B	A	B	November 2015	January/February 2016
1	19	15	14	13	2	7
2	16	12	13	18	3	Harvested
3	25	25	26	26	47	Harvested
4	33	30	27	24	64	Harvested
5	26	25	23	24	2	Harvested
6	27	28	23	19	13	Harvested
7	32	33	24	21	3	11
8	22	18	17	15	10	Harvested
9	18	21	18	14	14	Harvested
10	19	18	22	16	2	0
11	17	13	15	23	2	8
12	20	19	15	13	0	Harvested
13	13	20	20	17	5	Harvested
14	30	29	26	27	3	Harvested
15	35	32	26	26	46	45