# The Role of Terpenes as Protectors of the Potato Crop

This thesis is submitted in fulfilment of the requirements of the degree of Master of Science by Research at the University of East Anglia

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I declare that the work contained in this thesis, submitted by me for the degree of Master of Science by Research, is to the best of my knowledge my own original work, except where due reference is made.

Signed

**ABSTRACT**

Potato is the world's fourth largest food crop, however farmers lose a significant part of this crop due to factors such as disease and inefficient storage. Potato growers in North Norfolk, who grow specifically for the crisping industry, are under pressure to minimise losses.

Potato Cyst Nematode is a pathogen of the potato crop, affecting 80% of UK potato fields. The potato initiates infection by signalling its presence to nematodes. A heptacyclic triterpenoid, Solanoeclepin A, is released by potato roots into the soil. Nematodes sense this compound and infect the host. Possible solutions to mediate the problem require an understanding of the biosynthesis of Solanoeclepin A. Candidate oxidosqualene cyclase genes have been identified in potato and have been expressed in *N. benthamiana* to determine their function and possible involvement in Solanoeclepin A biosynthesis.

Major potato crop losses are also caused by the onset of sprouting during storage. Potatoes are harvested in September, but must be stored for up to ten months of the year. Chlorpropham (CIPC) is used widely in the industry as a sprouting inhibitor, however, EU legislation changes have reduced its usage. Monoterpenes may provide an alternative natural solution to sprouting inhibition. *S*-Carvone has been shown to suppress sprouting in potato varieties, and importantly, has no effect on processing quality of potatoes. Cyclodextrins have been studied as a possible mechanism for improving application efficiency of sprouting inhibitors. This work should be useful in providing alternative sprouting inhibitors for the potato industry.

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# List of Abbreviations

| | |
|---|---|
| **%** | Percent |
| **°C** | Degrees Celsius |
| **µg** | Microgram |
| **µl** | Microlitre |
| **µm** | Micrometre |
| **µM** | Micromolar |
| **ABA** | Abscisic acid |
| **bp** | Base pair |
| **CAS** | Cycloartenol synthase |
| **CIPC** | Isopropyl-3-chlorphenyl carbamate |
| **CK** | Cytokinins |
| **cm** | Centimetre |
| **CYP** | Cytochromes P450 |
| **DMN** | 1,4-dimethylnapthalene |
| **DNA** | Deoxyribonucleic Acid |
| **dNTP** | Deoxyribonucleotide triphosphate |
| **EST** | Expressed sequence tag |
| **EU** | European Union |
| **g** | Gram |
| **GA** | Gibberellins |
| **GC-MS** | Gas Chromatography – Mass Spectrometry |
| **GFP** | Green Fluorescent Protein |
| **HPβCD** | Hydroxypropyl-β-cyclodextrin |
| **J2** | Second-stage juvenile larva |
| **J3** | Third-stage juvenile larva |
| **J4** | Fourth-stage juvenile larva |
| **kbp** | Kilobase pair |
| **Kg** | Kilogram |
| **L** | Litre |
| **LB** | Lysogeny Broth |

| | |
|---|---|
| **LC-UV** | Liquid Chromatography – UV |
| **M** | Molar |
| **m/z** | Mass:Charge ratio |
| **MES buffer** | 2-(*N*-morpholino)ethanesulfonic acid |
| **mg** | Milligram |
| **ml** | Millilitre |
| **mm** | Millimetre |
| **mM** | Millimolar |
| **MS** | Murashige & Skoog medium |
| **ng** | Nanogram |
| **nm** | Nanometre |
| **$OD_{600}$** | Optical density (600 nm) |
| **OSC** | Oxidosqualene Cyclase |
| **p** | Probability |
| **PCN** | Potato Cyst Nematode |
| **PCR** | Polymerase Chain Reaction |
| **qRT-PCR** | Quantitative Reverse Transcription Polymerase Chain Reaction |
| **RNA** | Ribonucleic Acid |
| **rpm** | Revolutions per minute |
| **RT-PCR** | Reverse Transcription Polymerase Chain Reaction |
| **SPME** | Solid Phase MicroExtraction |
| **T6P** | Trehalose-6-Phosphate |
| **TMS** | Trimethylsilyl group |
| **TS** | Terpene synthase |
| **V** | Volt |
| **v/v** | Volume by volume |
| **w/v** | Weight by volume |
| **βAS** | β-amyrin synthase |
| **βCD** | β-cyclodextrin |

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# 1 Introduction

## 1.1 The Importance of the Potato

The potato – *Solanum tuberosum* - belongs to the Solanaceae family of flowering plants, which also includes species such as aubergine (*Solanum melongena*) and tomato *(Solanum lycopersicum*). *Solanum tuberosum* is a herbaceous annual plant that may grow up to 100 cm tall, and produces a tuber. The potato is the world's fourth most important food crop, following maize, wheat and rice. Whilst it is not clear from the evidence available when the potato was first introduced into Europe, it is believed to have been a cultivar originally growing in the Andes, which was introduced into Chile and then into Europe during the 16th Century (Hawkes 1992).

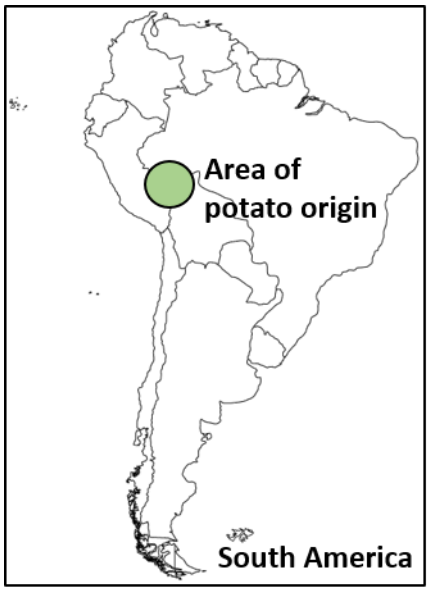


**Figure 1. Location of the origin of the potato.**

The importance of potato as a food crop is evident from the history of the plant and its introduction into non-native countries. Following the Spanish conquest of Peru in the 16th Century, the potato was taken to Spain, and from there spread throughout Europe. The potato was adopted as a major food crop in the northern hemisphere once varieties suited to long summer days were developed; this process took 150 years. The potato became the major food reserve of Europe during the Napoleonic wars, and has been referred to as the first modern 'convenience food' due to it being energy-rich, nutritious, easy to grow and cheap. However, the success of the potato in revolutionising food security in Northern Europe during this time also led to over-reliance on the crop, ultimately resulting in famine.

Only a few, genetically similar varieties were grown across North America and Europe meaning that it was a vulnerable crop, susceptible to pests and diseases. Late Blight (*Phytophthora infestans*), an oomycete, spread rapidly through the potato crops in continental Europe. Ireland was the worst affected, as the potato was relied on for 80% of the calorie intake at that time. From 1845-1848, three potato crops were destroyed by Late Blight, leading to death by famine for one million people (Nations 2009) Following this disaster, efforts were made to produce disease-resistant varieties, and breeders across Europe and North America drew on potato germplasm from Chile to produce modern varieties that have been responsible for the massive potato production throughout the 20th Century.

During the 20th Century, the potato finally emerged as a global food following its spread throughout Asia, and vast areas of arable land in Germany and Britain were dedicated to potato crops following the Second World War. Some countries, such as Belarus and Poland, still produce more potatoes than cereals. Today, modern inventions have revolutionised and vastly increased the ways in which potatoes are consumed. Mechanical potato peelers have led to potato crisps being America's top selling snack, and production facilities located worldwide mean that potatoes are consumed as French fries across the globe (Nations 2009).

## 1.2 Potato as a Crop

The fact that the world has become dependent on the potato as a major food crop means that the risks that the crop are exposed to need to be minimised to prevent future major crop failings, such as those that have happened in the past. Potato crops are still at risk from diseases such as Late Blight and Potato Cyst Nematode which can significantly impact on the yield of the potato crop. In addition, the demand for potatoes means that they must be available year round, as opposed to seasonally after harvest. Potatoes may be stored in cold facilities, however the biology of potatoes results in sprout growth after a period of time even in these conditions. Sprouted potatoes are unsuitable for the consumer, and therefore sprouting inhibition is required to enable potatoes to be stored year-round without sprouting occurring. Once potatoes have been removed from storage, they are destined either for the pre-pack or processing markets, and ultimately consumers will purchase either

the raw product or the processed goods. Farmers select which potato varieties to grow, and this is dependent on a number of factors including the productivity of the variety, disease resistance, length of storage period, and final destination of the crop, amongst many other factors. Clearly, farmers must be well informed as to the purpose of their crop, in order to ensure that it can be well maintained throughout the growing season, and to ultimately maximise yield and minimise profit losses.

Investigation into the factors affecting the potato crop yield is important to ensure the security of the potato crop, particularly in the face of the imminent global food security crisis. With the global human population expected to reach 9 billion by 2050, the demand for a constant, secure supply of food is ever increasing, and therefore crop productivity needs to be improved in order to fulfil this demand. Crop yield losses can be minimised in a variety of ways including prevention of loss through disease and post-harvest storage and processing.

## 1.3 Plant Secondary Metabolism

Plants are continually under attack from a variety of pathogens, however plants must protect themselves against such pathogenic attack for survival. Despite the fact that pathogens inhabit the same environment as plants, plants are rarely colonised by these pathogens. This is due to the plants immune system. Plant secondary metabolites: compounds not essential to the plants core metabolism, are widely accepted to contribute to the interaction between plants and other organisms (Hartmann 2008). A vast array of secondary metabolites with huge diversity in structure have proven or putative functions in plant protection against pathogenic microorganisms and these have largely been reviewed by Piasecka *et al*. (2015) (Piasecka, Jedrzejczak-Rey et al. 2015). There are two classes of defensive metabolites: the phytoanticipins and the phytoalexins. Phytoanticipins are compounds present in plants before challenge by microorganisms, whilst phytoalexins are produced in response to a microbial elicitor (VanEtten, Mansfield et al. 1994). The main classes of plant secondary metabolites include: alkaloids, glucosinolates and cyanoglucosides, phenylpropanoids and other phenolic compounds, and terpenoids (Piasecka, Jedrzejczak-Rey et al. 2015).

Plant secondary metabolites may be produced by plants as a component of the phenomenon known as allelopathy. Allelopathy is the process in which a plant may produce a chemical or

variety of chemicals in order to influence the growth, survival or reproduction of other organisms (Rizvi, Haque et al. 1992). This may be beneficial to the plant as it allows manipulation of other organisms in the environment, in order to promote the plants own survival. Examples of such chemical compounds include metabolites for defence against pathogen attack, along with metabolites inhibiting the growth of other species in the environment. Interestingly, such metabolites may be exploited, both by pathogens, which may use these metabolites to their advantage for signalling purposes, and by humans for the development of pesticides and methods of controlling plant growth.

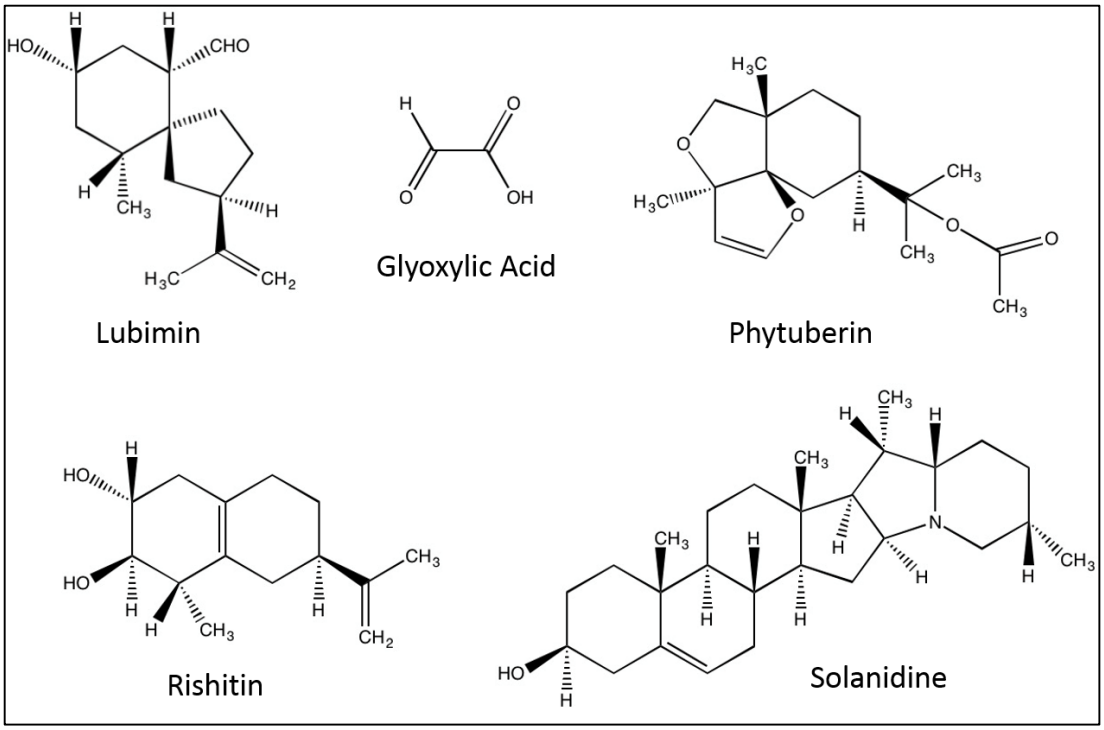


**Figure 2. Toxins found in potato (*Solanum tuberosum*). (Baxter 1996)**

In potato there are a number of secondary metabolites as seen in Figure 2, including the steroidal glycoalkaloids. α-solanine, a steroidal glycoalkaloid, is a toxic metabolite found in potatoes, that is thought to protect the plant against herbivory (Itkin, Heinig et al. 2013), however Solanoeclepin A – a related terpene – is an example of a compound exploited by the Potato Cyst Nematode for hatching (Mulder, Diepenhorst et al. 1996). This therefore demonstrates how related compounds can be both protective yet also be exploited by a pathogen. Solanoeclepin A will be discussed in Chapter 3 of this thesis.

## 1.4 Terpenes

Terpenoids constitute the largest and most widespread group of plant secondary metabolites, and they display immense structural diversity. Terpenoids are formed from linear arrangements of isoprene units, and are then rearranged and cyclised in order to form the terpenoid carbon skeleton. Terpenoids are classified according to the number of isoprene units from which they are constructed, and these classes include monoterpenes ($C_{10}$), sesquiterpenes ($C_{15}$), diterpenes ($C_{20}$), triterpenes ($C_{30}$) and tetraterpenes ($C_{40}$) to name just a few (Stevens 1992). Terpene synthases, a large class of enzymes, are responsible for the diversity observed in terpene carbon skeleton structures. The diversity in terpene structure is also due to the fact that some terpene synthases produce multiple products (Degenhardt, Köllner et al. 2009). Investigation into terpene synthases has been an active area of plant metabolic engineering research in recent years due to the fact that the vast diversity of terpenes may be exploited for biotechnological purposes (Singh and Sharma 2015). These compounds have an array of interesting properties, which may be exploited for medicinal and agrochemical purposes, as well as for flavouring and in cosmetics. As the field of synthetic biology develops, terpenes may be interesting and useful compounds to investigate and exploit, due to their high level of diversity and the wide array of industries to which they may be applied.

Terpenes have been identified in this study as being associated with potato crop losses in a variety of different manners. The compound identified as a 'hatching factor' for Potato Cyst Nematodes, which stimulates nematodes to hatch in the presence of the host plant, is a triterpenoid produced by potatoes. Understanding of the ecological role and biosynthesis of this triterpenoid will shed light on the reason for the production of this 'hatching factor' by the potato, and may also be critical in identifying solutions to the problem of Potato Cyst Nematode infection.

Monoterpenes have been identified in this study as having a role in potato sprouting suppression. *S*-Carvone, a monoterpene, is suggested to have inhibitory properties for the process of potato sprouting. The use of plant-derived metabolites for the suppression of potato sprouting on an industrial scale is of interest as large losses of the crop result from sprouting whilst in storage, however inhibitors currently used are subject to legislation changes. Therefore, an alternative, safe and environmentally-friendly sprouting inhibitor is required in order to prevent costly crop losses as a result of potato sprouting.

Terpenes have therefore been investigated in this study as potential 'protectors' of the potato crop.

## 1.5 Aims

This study aims to investigate two of the major factors affecting potato crop yields. Potato Cyst Nematode, a major pathogen of the potato crop, will be investigated in order to identify potential biological solutions for this problem.

Secondly, potato tuber sprouting in storage will be investigated in order to find potential new inhibitors for the potato industry, particularly for the potato processing market. Methods for improving conventional sprouting inhibitor treatments will be investigated, along with alternative inhibitors, in particular natural products.

Terpenes have been identified as possible candidates for mitigating the potato crop yield losses caused by these two problems, and therefore will be investigated to determine their potential roles in securing the future of the crop.

# 2 Materials and Methods

## 2.1 Materials and Methods – Heterologous Expression of Oxidosqualane Cyclases in *Nicotiana benthamiana*

### 2.1.1 Materials

All materials were sourced from Sigma-Aldrich, unless stated otherwise. Cyclodextrins were sourced from CycloLabs.

#### 2.1.1.1 Bioinformatic Analysis and Computational Software

The SpudDB genome database (*Solanum tuberosum*) and NCBI database was used for identification of Oxidosqualene Cyclases (OSC) homologs in potato. MEGA6 software (Tamura, Stecher et al. 2013) and ClustalW (Larkin, Blackshields et al. 2007) were used for alignment and analysis of phylogenetic relationships. BioEdit (Hall 1999) was used for the analysis of experimental sequence data, and Primer3 (Untergasser, Cutcutache et al. 2012) software was used for the design of expression profiling primers. Eurofins Oligo analysis tool was used to determine primer properties. Statistical analysis was carried out using GenStat software (Committee 2013).

#### 2.1.1.2 Plant Material and Growth Strains

Potato variety Desiree plant was grown axenically on MS (Murashige and Skoog) medium + 2% sucrose, pH 5.7 in a growth chamber (22 $^{o}$C; 14 hour day, 10 hour night). Potato samples were recultured on a 4 week cycle. Potato tissue was originally acquired from The Sainsbury Laboratory (Norwich).

#### 2.1.1.3 Plasmid Constructs

**Table 1. Plasmid constructs.**

| Plasmid | Resistance | Vector Type |
|---|---|---|
| PCR8/GW | Spectinomycin | Cloning Vector |
| pDONR207 | Gentamycin | Cloning Vector |
| pEAQ-HT-DEST2 | Kanamycin | Expression Vector |

### 2.1.1.4 Primers

**Table 2. Primer sequences used for cloning and expression profiling.**

| Primer Pair (F/R sequence) | Sequence | Length of amplicon (bp) cDNA/gDNA | Tm |
|---|---|---|---|
| β-amyrin synthase1 | 5'-ATGTGGAAATTGAAGATTGCTGAAGGG-3'<br>3'-TTAGTTGTTTTCTAATGGTAATAGGAC-5' | 2286 bp | 63 °C<br>58 °C |
| β-amyrin synthase2 | 5'-ATGTGGAAGTTGAAGATTGCAAAAGGAC-3'<br>3'-TTAGTTGTGTACTAATGGTACTTGGAC-5' | 2202 bp | 63 °C<br>61 °C |
| β-amyrin synthase internal primer | 5'-TGTCACCGCAATTCCTTCTTGG-3'<br>3'-ATTCACCGAGTCATACAGTCG-5' | For sequencing | 63 °C<br>61 °C |
| Cycloartenol synthase (truncated) | 5'-ATGTGGTGCCATTGTCGTATGG-3'<br>3'-TCATTGAGGGTTAAGTAGCTGAGAC-5' | 1515 bp | 63 °C<br>63 °C |
| Cycloartenol synthase (full length) | 5'-ATGTGGAAGTTGAAGGTTGCTGAAGG-3'<br>3'-TCATTGAGGGTTAAGTAGCTGAGACTG-5' | 2274 bp | 64 °C<br>65 °C |
| Cycloartenol synthase + gateway flanking regions | 5'-ggggacaagtttgtacaaaaaagcaggcttaatgATGTGGAAGTTGAAGG-3'<br>3'-ggggaccactttgtacaagaaagctgggtaTCATTGAGGGTTAAGTAGC-5' | 2335 bp | 72 °C<br>74 °C |
| **Expression Profiling primers** | | | |
| β-amyrin synthase1 | 5'-GGTCATTGGCCTGCTGAAAA-3'<br>3'-GGAATTGCGGTGACACTACC-5' | 314 bp/796 bp | 59 °C<br>59 °C |
| Delta-amyrin synthase | 5'-GTGCGCCATGTATGTGCTAA-3'<br>3'-TGACTACCACAACCCTGCAT-5' | 153 bp/153 bp | 59 °C<br>59 °C |
| Cycloartenol Synthase | 5'-GCTTGGCCTTTCTCTACTGC-3'<br>3'-CATATGTCCCAATGCCACCG-5' | 193 bp/1020 bp | 59 °C<br>59 °C |
| Lupeol Synthase | 5'-GTGCCGATTTACTCATGCGT-3'<br>3'-GAAGAATAAAGGGCCGGCAG-5' | 200 bp/317 bp | 59 °C<br>59 °C |
| GAPDH | 5'-GGTTGTGATCTCCGCTCCTA-3'<br>3'-CCACAATGCCAAACCTGTCA-5' | 161 bp/161 bp | 55 °C<br>50 °C |

Primers were designed using the Eurofins Oligo Analysis Tool to determine the properties of each sequence, and were synthesised by Eurofins Genomics.

Expression profiling primers were designed using the Primer3 automatic primer generator. Lower case letters = gateway flanking regions.

### 2.1.1.5 Bacterial Strains

**Table 3. Bacterial strains used for cloning and expression.**

| **Bacterial strains** |
|---|
| *E. coli* TOP10 |
| *Agrobacterium tumefasciens* (LBA4404) |

### 2.1.1.6 Media and antibiotics

Lysogeny broth (LB) was used to grow all bacterial cultures, both in liquid shaking form and on plates.

Antibiotics were added to the media for resistance selection. Antibiotics were added in the following concentrations:

**Table 4. Antibiotics used for antibiotic resistance screening.**

| **Antibiotic** | **Stock Concentration** | **Final Concentration** |
|---|---|---|
| Gentamycin | 10 mg/mL | 10 µg/mL |
| Kanamycin | 50 mg/mL | 50 µg/mL |
| Rifampicin | 50 mg/mL | 100 µg/mL |
| Spectinomycin | 100 mg/mL | 100 µg/mL |
| Streptomycin | 100 mg/mL | 100 µg/mL |

### 2.1.2 Methods

#### 2.1.2.1 Molecular Biology

##### *2.1.2.1.1 RNA extraction*

Potato tissue samples were flash frozen in liquid nitrogen before being ground using a pestle and mortar to a fine powder. 30 mg ground tissue was used for RNA extraction. RNA was then extracted using the Promega SV Total RNA Isolation System kit. RNaseZAP (Sigma-Aldrich) was used to remove contamination from RNase. RNA was stored at -80 °C following extraction, and concentration was determined using Nanodrop (NanoDrop ND-1000). This RNA was then used both for RT-PCR and cDNA synthesis.

##### *2.1.2.1.2 DNA extraction*

Potato tissue samples were flash frozen in liquid nitrogen before being ground using a pestle and mortar to a fine powder. 40 mg ground tissue was used for DNA extraction. DNA was extracted following the Promega Wizard Genomic DNA Purification kit. Extracted DNA was stored at -20 °C following extraction. Extracted genomic DNA was used for expression profiling experiments.

##### *2.1.2.1.3 cDNA synthesis*

cDNA was synthesised using extracted RNA as template using the Invitrogen SuperScript 2 Reverse Transcriptase kit. Oligo(dT)s (500 μg/mL) were used along with this kit. cDNA concentration was determined using Nanodrop.

##### *2.1.2.1.4 Gene Synthesis*

Genes were synthesised by IDTDNA using the gBlocks Gene Fragments service provided for synthesising genes smaller than 2000 base pairs. Fragments were delivered as double stranded DNA.

### *2.1.2.1.5 Gibson Assembly*

Gibson Assembly Master Mix (NEB) was used to assemble synthesised DNA fragments together to form desired DNA sequence.

**Table 5. Gibson Assembly protocol.**

| **2 Fragment Assembly** | **20 µl reaction** |
|---|---|
| Fragment A (0.02 – 0.5 pmols x µl) | 2.5 µl |
| Fragment B (0.02 – 0.5 pmols x µl) | 2.5 µl |
| Gibson Assembly MasterMix 2X | 10 µl |
| Nuclease-Free Water | 5 µl |

This reaction was then incubated in a thermocycler at 50 °C for 30 minutes before being stored at –20 °C. This template could then be used as a template for PCR.

### *2.1.2.1.6 Polymerase Chain Reaction (PCR)*

#### Reverse-Transcriptase PCR

OneStep RT-PCR Kit (Qiagen) was used for RT-PCR, during which reverse transcription and PCR are carried out sequentially in the same tube. Therefore, template used in this reaction was RNA as opposed to DNA. RNA must be kept on ice in order to prevent degradation. RNA was stored at -80 °C.

**Table 6. RT-PCR protocol.**

| **Reagent** | **50 µl reaction** | **Final Concentration** |
|---|---|---|
| 5X Qiagen OneStep RT-PCR Buffer | 10 µl | 1X |
| 10 mM dNTPs | 2 µl | 0.4 mM |
| 6 µM Forward Primer | 5 µl | 0.6 µM |
| 6 µM Reverse Primer | 5 µl | 0.6 µM |
| Template RNA | ~1 µg | ~1 µg |
| Qiagen OneStep RT-PCR Enzyme mix | 2 µl | - |
| RNase-Free water | To 50 µl | - |

PCR thermo-cycle programme

**Table 7. RT-PCR thermo-cycle programme.**

| Step | Temperature | Time |
|---|---|---|
| Reverse Transcription | 50 °C | 30 minutes |
| Initial PCR Activation | 95 °C | 15 minutes |
| 30 cycles | 94 °C<br>50-68 °C *<br>72 °C | 1 minute<br>1 minute<br>2 minutes |
| Final Extension | 72 °C | 10 minutes |
| Hold | 10 °C | - |

Annealing temperature was altered dependent on melting temperature of primers used (*). Annealing temperature = Primer melting temperature – 5 °C.

## Q5 Hi-FID PCR

A high fidelity polymerase was used for accurate amplification of cDNA template. Q5 High-Fidelity DNA Polymerase (NEB) was used in the following reaction set up:

**Table 8. Q5 Hi-Fid PCR protocol.**

| Reagent | 50 µl reaction | Final Concentration |
|---|---|---|
| 5X Q5 Reaction Buffer | 10 µl | 1X |
| 10 mM dNTPs | 1 µl | 200 µM |
| 10 µM Forward Primer | 2.5 µl | 0.5 µM |
| 10 µM Reverse Primer | 2.5 µl | 0.5 µM |
| Template DNA | <1000 ng | <1000 ng |
| Q5 High-Fidelity DNA Polymerase | 0.5 µl | 0.02 U/µl |
| Nuclease-Free water | To 50 µl | - |

PCR thermo-cycle programme

**Table 9. Q5 Hi-Fid PCR thermo-cycle programme.**

| Step | Temperature | Time |
|---|---|---|
| Initial Denaturation | 98 °C | 30 seconds |
| 30 cycles | 98 °C<br>50-72 °C *<br>72 °C | 10 seconds<br>30 seconds<br>1.5 minutes (30 seconds/kb) |
| Final Extension | 72 °C | 2 minutes |
| Hold | 10 °C | - |

Annealing temperature was altered dependent on melting temperature of primers used (*). Annealing temperature = Primer melting temperature – 5 °C.

## Go-Taq MasterMix PCR

GoTaq Green G2 MasterMix Polymerase (Promega) used in expression profiling PCR.

**Table 10. Go-Taq MasterMix PCR protocol.**

| Reagent | 50 µl reaction | Final Concentration |
|---|---|---|
| GoTaq G2 Green MasterMix 2X | 25 µl | 1X |
| 10 µM Forward Primer | 2 µl | 0.4 µM |
| 10 µM Reverse Primer | 2 µl | 0.4 µM |
| DNA template | <250 ng | <250 ng |
| Nuclease-Free water | To 50 µl | - |

PCR thermo-cycle programme

**Table 11. Go-Taq MasterMix PCR thermo-cycle programme.**

| Step | Temperature | Time |
|---|---|---|
| Initial Denaturation | 95 °C | 2 minutes |
| 30 cycles | 95 °C<br>52 °C<br>72 °C | 30 seconds<br>45 seconds<br>1 minute |
| Final Extension | 72 °C | 5 minutes |
| Hold | 10 °C | - |

## Colony PCR

*E. coli* colonies were picked off the antibiotic selection plates once grown and colony PCR carried out to check the insertion of the desired gene into the vector that *E. coli* cells were subsequently transformed with. 10 colonies were picked off the plate and re-suspended in autoclaved water. The cell suspensions were boiled (100 °C) for 20 minutes on a heating block, and then used in the following PCR reaction mixture.

**Table 12. Colony PCR protocol.**

| **Reagent** | **50 µl reaction** |
|---|---|
| 10x Standard *Taq* Reaction Buffer | 5 µl |
| 10 mM dNTPs | 1 µl |
| 10 µM Forward Primer | 1 µl |
| 10 µM Reverse Primer | 1 µl |
| Colony Suspension | 10 µl |
| *Taq* DNA Polymerase | 0.25 µl |
| Nuclease-free water | 31.75 µl |

PCR thermo-cycle programme

**Table 13. Colony PCR thermo-cycle programme.**

| **Step** | **Temperature** | **Time** |
|---|---|---|
| Initial denaturation | 95 °C | 30 seconds |
| 30 cycles | 95 °C<br>55 °C *<br>68 °C | 20 seconds<br>30 seconds<br>2.5 minutes |
| Final extension | 68 °C | 5 minutes |
| hold | 10 °C | - |

The annealing temperature (*) of the PCR programme was adjusted dependent on the melting temperature of primers being used. Primers used in colony PCR reactions included a plasmid specific primer and a gene specific primer in order to screen for and eliminate colonies which may have the gene inserted in the wrong orientation, particularly in the case of TOPO cloning (non-directional).

#### *2.1.2.1.7 PCR purification*

PCR products were purified using the QiaQuick PCR Purification kit (Qiagen), in order to remove buffers from reaction mix before using in later cloning steps. DNA was eluted in 40 µl nuclease free $H_2O$.

#### *2.1.2.1.8 Agarose Gel Electrophoresis*

1% agarose gels were used to analyse DNA and RNA samples (unless otherwise stated). 500 mg agarose was added to 50 mL TAE buffer. The solution was heated by microwave for 1 minute in order for the agar to dissolve. After cooling, 5 µl SYBR Safe DNA gel stain (Invitrogen) was added. Agarose was poured into electrophoresis tray with comb and allowed to set. The gel was then placed in an electrophoresis tank and covered with TAE buffer. 6X DNA loading dye was added to DNA samples in a ratio 1:5 and loaded into wells in the gel. DNA ladders (50 bp, 100 bp, 1 kb or 2-Log) were added, and the gel was run at 110 V until the loading dye was approximately half way down the gel. Gel was them imaged using the UV setting on SynGene G-Box gel imager.

#### *2.1.2.1.9 Gel Extraction*

PCR bands were extracted from Agarose gels following electrophoresis in order to further purify the PCR product and to ensure that only the band of interest (corresponding to the DNA ladder) was used in later cloning steps. Gel extraction was carried out using the QiaQuick Gel Extraction kit (Qiagen). DNA was eluted in 40 µl nuclease free $H_2O$.

### 2.1.2.2 Cloning Techniques

#### *2.1.2.2.1 3' A-overhang addition*

Following amplification with high fidelity polymerase, PCR products to be used in TOPO cloning require the addition of 3' A overhang with *Taq* polymerase in order for insertion into the vector to occur. This mixture was then incubated at 72 °C for 30 minutes.

**Table 14. 3' A-overhang addition protocol.**

| **Reagent** | **50 µl total** |
|---|---|
| 10x Standard Taq Buffer | 5 µl |
| dATP (10mM) | 1 µl |
| 0.5 U *Taq* (5 U/µl) | 0.1 µl |
| DNA | 40 µl |
| Nuclease Free $H_2O$ | 3.9 µl |

### *2.1.2.2.2 pCR8/GW/TOPO TA Cloning*

In order to insert the PCR product into the pCR8 vector, the following reaction mixture was set up. This was incubated at 23 °C for at least 2 hours in order to allow for efficient insertion of PCR product into vector.

**Table 15. pCR8/GW/TOPO cloning protocol.**

| **Reagent** | **6 µl total** |
|---|---|
| PCR product | 3 µl |
| Salt solution | 0.5 µl |
| Water | 2 µl |
| TOPO (pCR8 vector) | 0.5 µl |

### *2.1.2.2.3 Gateway Technology*

Gateway Technology can be used to ligate DNA into desired vectors. Gateway Technology is based on the site-specific recombination of the bacteriophage lambda into the *E. coli* chromosome (Invitrogen 2003). Recombination occurs between specific attachment (*att*) sites on the DNA molecules to undergo recombination, and strand exchange occurs between homologous 15 bp core regions in the *att* sites. Clonase enzyme mixes mediate the recombination reaction (Invitrogen 2003).

#### Gateway Technology – LR reaction

Gateway Technology (Invitrogen) was used to ligate the desired gene present in the entry vector into the destination vector (pEAQ-*HT*-DEST2) (Sainsbury, Thuenemann et al. 2009). The LR reaction facilitated the recombination of an *att*L substrate (entry clone – pCR8/GW vector) with the *att*R substrate (destination vector).

**Table 16. Gateway cloning - LR reaction.**

| **Reagents** | **Reaction Mixture (8 µl)** |
|---|---|
| pCR8/GW with gene insert (entry) Clone | 50-150 ng |
| pEAQ-H*T*-DEST2 (destination) vector | 150 ng |
| TE Buffer (pH 8.0) | Up to 8 µl |

2 µl LR Clonase 2 (Invitrogen) was thawed on ice and vortexed briefly. 2 µl LR Clonase 2 was added to the reaction mixture described and mixed. The reaction was incubated at 25 °C for 1 hour, before 1 µl Proteinase K was added and incubated at 37 °C for 10 minutes to terminate the reaction. This ligation mixture was used to transform *E. coli* cells (described section 1.4.2), which were then grown on LB + kanamycin plates.

### Gateway Technology – BP reaction

Gateway Technology (Invitrogen) was used to ligate the PCR amplified DNA into the donor vector (pDONR207). The BP reaction facilitated the recombination of an *att*B substrate (*att*B-PCR product) with the *att*P substrate (donor vector).

**Table 17. Gateway cloning - BP reaction.**

| Reagents | Reaction Mixture (8 µl) |
|---|---|
| *att*B-PCR product | 150 ng |
| pDONR207 (donor) vector | 150 ng |
| TE Buffer (pH 8.0) | Up to 8 µl |

2 µl BP Clonase (Invitrogen) was thawed on ice and vortexed briefly. 2 µl BP Clonase was added to the reaction mixture described and mixed. The reaction was incubated at 25 °C for 1 hour, before 1 µl Proteinase K was added and incubated at 37 °C for 10 minutes to terminate the reaction. This ligation mixture was used to transform *E. coli* cells (described section 1.4.2), which were then grown on LB + gentamycin plates.

### 2.1.2.3 Cell Transformation and Sequencing

#### *2.1.2.3.1 Competent Cell Preparation*

For the preparation of electrocompetent cells, LB starter cultures were inoculated and grown overnight at 37 $^{o}$C whilst shaking. Overnight cultures were subcultured in 50 ml fresh LB media until reaching an $OD_{600}$ = ~0.3. Cultures were centrifuged at 4 $^{o}$C, 4000 rpm for 10 minutes and resuspended in cold water. Cells were centrifuged again and 10% glycerol (sterile) was added, before being centrifuged again. Glycerol was removed, and a small amount of 10% glycerol was added. Cells were aliquot out and stored -80 $^{o}$C.

#### *2.1.2.3.2 Transformation of E. coli cells*

**Using Electroporation**

To prevent arcing of cells due to high salt concentration, the reaction mixture was diluted 4-fold (ratio water to TOPO cloning reaction 3:1) (TOPO cloning only). 3 µl of diluted reaction mixture was added to 50 µl electrocompetent *E. coli* cells. Cells were then transferred to a 2 mm Geneflow electroporation cuvette and were electroporated using Bacteria settings on BioRad Micropulser electroporation machine.

**Using Chemical Transformation**

3 µl of the reaction mixture was added to 50 µl chemically competent *E. coli* cells and mixed gently. This mixture was incubated on ice for 30 minutes before being heat-shocked at 42 °C for 30 seconds. Following heat-shock, the cells were placed back on ice immediately.

### *2.1.2.3.3 Cell Recovery and Antibiotic Resistance Expression*

250 µl LB media was added to the cells immediately following transformation (electroporation or chemical), and this Eppendorf was left to shake at 37 °C for 1 hour to allow expression of antibiotic resistance gene. Following incubation, cells were spread onto antibiotic selective plates in two different volumes (50 µl, 200 µl) to ensure well spread colonies on at least one plate. Plates were incubated at 37 °C overnight to allow for transformed *E. coli* colonies to grow. Plates were then stored at 4 °C.

### *2.1.2.3.4 Transformation of competent Agrobacterium tumefasciens cells*

Following the extraction of pEAQ-*HT*-DEST2 destination vector from *E. coli*, competent *A. tumefasciens* (strain LBA4404) cells were transformed with this vector using a cold shock protocol. An aliquot of competent cells was thawed on ice for approximately 2 hours. 100-200 ng plasmid DNA was added to the competent cells and mixed gently. This was placed in liquid nitrogen for 1 minute and then thawed at room temperature.

***Agrobacterium tumefasciens* Cell Recovery and Antibiotic Resistance Expression**

200 µl SOC media was added for recovery and this mixture was incubated at 28 °C for 3 hours. 200 µl of these cells were plated onto LB + rifampicin + kanamycin + streptomycin selection plates and left to grow at 28 °C for 3 days.

Once grown, colonies were inocculated in 50 ml LB + rifampicin + kanamycin + streptomycin Erlenmeyer flasks and grown in a shaking incubator at 28 °C overnight.

### *2.1.2.3.5 Isolation of Plasmid DNA from E. coli*

Colonies screened by colony PCR and determined to have the correct size gene insert were used to inoculate 5 ml liquid LB + appropriate antibiotic cultures, which were then grown in a 37 °C shaking incubator overnight. Plasmid DNA was then extracted from *E. coli* cells using the Qiagen Spin Miniprep kit. 5 ml *E. coli* overnight cultures were pelleted by centrifugation at 4000 rpm for 8 minutes at room temperature. The pelleted cells were resuspended in 250 µl Buffer P1 and 250 µl Buffer P2 (lysis buffer) was added. 350 µl Buffer N3 was added and mixed, and this suspension was then centrifuged for 10 minutes at 13000 rpm in a table-top microcentrifuge.

The supernatant was applied to the membrane of a spin column, and washed using 500 µl Buffer PB and 750 µl Buffer PE. Residual wash buffer was removed by centrifugation and the DNA was then eluted using 40 µl nuclease-free water.

### *2.1.2.3.6 DNA Sequencing*

Extracted plasmids were sequenced to ensure the inserted gene had the desired sequence. Big Dye 3.1 (Life Technologies – Invitrogen) was used to carry out the sequencing reaction as follows:

**Table 18. DNA sequencing protocol.**

| **Reagent** | **10 µl reaction mix** |
|---|---|
| Big Dye Buffer | 2 µl |
| Big Dye 3.1 | 1 µl |
| Plasmid (~100 ng/µl) | 1 µl |
| Primer (10 µM) | 1 µl |
| Water | 5 µl |

Primers used for sequencing included forward and reverse plasmid specific primers, and internal gene specific forward and reverse primers. Therefore, four sequencing reactions were set up per plasmid sequenced in order to ensure full coverage of the gene (~2.4 kb). The reaction heat cycle programme was set up as follows:

**Table 19. DNA sequencing thermo-cycle programme.**

| **Big Dye reaction cycle (25 cycles)** | **Time** |
|---|---|
| 96 °C | 10 seconds |
| 50 °C | 10 seconds |
| 60 °C | 4 minutes |
| 10 °C Hold | - |

Samples were transferred to 1.5 ml eppendorfs and sent to Eurofins MWG for sequencing. Data files were subsequently analysed in BioEdit.

### 2.1.2.4 Expression of Genes *in planta*

#### *2.1.2.4.1 Infiltration and Transient Expression of Genes in Nicotiana benthamiana*

Overnight *A. tumefasciens* cultures were centrifuged at 4000 rpm for 10 minutes and the pellet was re-suspended in 5 mL MMA solution and incubated in the dark at room temperature for at least 1 hour.

**Table 20. MMA solution.**

| MMA solution | Final Concentration | Stock Solution | 100 mL |
|---|---|---|---|
| $MgCl_2$ | 10 mM | 1 M | 1 mL |
| MES/KOH pH 5.6 | 10 mM | 1 M | 1 mL |
| Acetosyringone | 150 µM | 150 mM | 100 µL |
| Milli-Q water | - | - | 97.9 mL |

$OD_{600}$ was checked – 100 µL culture suspension was diluted in 900 µL MMA solution in a cuvette; the OD reading was multiplied by 10 to ensure an accurate reading was obtained. The culture was diluted in MMA solution in order to obtain an OD of 0.2. This OD value has been determined to ensure that a copy of the gene is delivered to every cell.

For infiltration of *N. benthamiana* leaves, plants should be 3 weeks old. Small holes were made on the underside of the leaves to be infiltrated using a sterile pipette tip and *A. tumefasciens* culture was infiltrated into the leaf using a 5 mL needless syringe. Plants were left to grow for 6 days in glass houses.

#### *2.1.2.4.2 Extraction of Triterpenes from Nicotiana benthamiana*

*N. benthamiana* leaves were collected approximately 6 days after agro-infiltration and the leaves stored at -80 °C until use. 1 cm diameter disks were cut from leaves and added to the saponification mix (200-300 µL/leaf disk).

**Table 21. Saponification mix.**

| Reagent – Saponification Mix | Amount (w/v or v/v) |
|---|---|
| Ethanol | 9 parts |
| Water | 1 part |
| KOH Pellets | 1 part |

The leaf disk in saponification mix was heated at 65 °C for 2 hours, shaking intermittently, before the addition of 50 µL water. 500 µL hexane was added to partition the mix, before the hexane layer was removed and transferred to a new vessel. Hexane was dried under $N_2$ and re-suspended in 100 µL derivatising reagent (TMS imidazole). Samples were heated to 70 °C for 30 minutes, and were then ready for analysis by Gas Chromatography – Mass Spectrometry (GC-MS) (Geisler et al. 2013).

### 2.1.2.5 Analysis by Gas Chromatography-Mass Spectrometry

Analysis of extracted triterpenes was carried out using the GC-MS system in the Metabolomics Department, John Innes Centre. The column used for analysis was a ZB-5HT column (30 m + 5 m guard column, 0.1 µm film thickness). Splitless injection was used with a carrier gas flow of 1.0 ml/minute. The quadrupole was set in scan mode, and scanned masses from 60 to 800. A solvent delay of 15 minutes was included in the method. Chemical standards were analysed by GC-MS to confirm the presence of compounds in the sample – cycloartenol standard was sourced from Sigma, and β-amyrin standard was sourced from Extrasynthese.

**Table 22. GC-MS temperature cycle programme for triterpene analysis.**

| **Temperature Cycle** | **Temperature** | **Time** |
|---|---|---|
| Injection Temperature | 250 °C | - |
| Initial Oven Temperature | 170 °C | 2 minutes |
| Ramp 1 | 170 °C – 290 °C<br>Hold 290 °C | 6 °C/minute<br>4 minutes |
| Ramp 2 | 290 °C – 340 °C | 10 °C/minute |

Ionisation method for mass spectrometry used electron ionisation (EI) at 70 eV.

## 2.2 Materials and Methods - Potato Sprouting Inhibition in Cold Box storage

### 2.2.1 Potato Varieties and Storage Conditions

Potato varieties Hermes, Lady Claire and Markies were used for sprouting assessment. (Hermes: short term storage, Lady Claire: medium term storage, Hermes: long term storage).

**Table 23. Potato varieties used.**

| Variety | Grower | Irrigated/Non-irrigated Field | Original Storage Facility |
|---|---|---|---|
| Hermes | FJC | Irrigated | Rookery 3 |
| Lady Claire | CFL | Irrigated | Swayfield |
| Markies | CFL | Irrigated | CEX5 |

The three varieties were specifically grown for crisp production, therefore varieties suitable for this purpose were selected. The three varieties were grown in irrigated fields in the North Norfolk region during the 2014 growing season, and harvested in September 2014. The potatoes were stored in box storage facilities at 9 °C for two weeks to allow for wound healing, and were then moved to a small scale cold box storage facility, held at 11 °C and 90% humidity on 13/10/2014. This temperature was gradually dropped to 9 °C by 15/01/2015. Soil was removed from potato tubers using a scrubbing brush to remove dried dirt prior to treatment, to enable accurate measurement of sprout development, and to ensure direct application of compounds tested.

**Figure 3. Nelson County Potatoes field locations in North Norfolk.**

### 2.2.2 Treatment Compounds

Chlorpropham (isopropyl-3-chlorophenyl carbamate, CIPC) (Sigma-Aldrich) and *S*-Carvone (Sigma-Aldrich) were tested as sprouting inhibitors. 2-hydroxypropyl-β-cyclodextrin (HPβCD) (Cyclolabs) and β-cyclodextrin (βCD) (Cyclolabs) were also tested in complex with sprouting inhibitors. CIPC was conventionally applied as a hot fog in the mass storage facility, whereas all other treatments were applied as an aqueous spray. Comparison between conventional hot fog application and aqueous spray of CIPC allowed for the difference in efficiency of application method to be taken into account.

Conventional hot fogging treatment took place two weeks after potatoes put into store (19.10.2014) whereas first aqueous spray treatment took place later (13.11.2014) due to logistical reasons. For this reason, aqueous spray application was carried out two weeks after each hot fog CIPC application in order for the time between applications to be equal. Equivalent quantitities of CIPC (12 g/tonne) were used to treat both the conventionally treated potatoes and aqueous spray treated potatoes at each application.

**Table 24. Dates of treatment application.**

| Treatment | Untreated | CIPC (Hot Fog) | CIPC (Wet Spray) | HPβCD | CIPC x HPβCD | S-Carvone (+ 1% ethanol) | βCD | S-Carvone x βCD (+ 1% ethanol | 1% ethanol |
|---|---|---|---|---|---|---|---|---|---|
| 1 | - | 19.10.14 | 13.11.14 | 13.11.14 | 13.11.14 | 13.11.14 | 13.11.14 | 13.11.14 | 13.11.14 |
| 2 | - | 13.01.15 | 04.02.15 | 04.02.15 | 04.02.15 | 16.01.15 | 16.01.15 | 16.01.15 | 16.01.15 |
| 3 | - | 14.03.15 | 24.03.15 | 24.03.15 | 24.03.15 | 18.03.15 | 18.03.15 | 18.03.15 | 18.03.15 |
| 4 | - | 02.05.15 | 13.05.15 | 13.05.15 | 13.05.15 | 13.05.15 | 13.05.15 | 13.05.15 | 13.05.15 |

### 2.2.3 Application Method

100 potato tubers of the three varieties were allocated for each treatment. Therefore, 2700 potato tubers were used for this trial. Tubers were placed into trays (H: 8.9 cm, L: 59.8 cm, W: 39.7 cm), each holding 50 tubers. Two trays per variety were treated with each compound being tested. Tuber tray weight was recorded at the start of the trial, and at regular intervals throughout the trial.

A 1:1 molar ratio of CIPC to HPβCD was used for complex formation, and equal quantities of both CIPC and HPβCD alone were also used as treatments. Sufficient treatment compound was prepared for 45 kg of potato tuber in 50 mL water. Samples were heated to 60 °C in a water bath and sonicated in a sonication bath for 15 minutes in order to improve solubility. Samples were prepared 24 hours prior to treatment and were left at room temperature overnight in order to allow complexation between CIPC and HPβCD to occur. The quantity used to treat each sample was calculated relative to potato tray weight. Mass (of treatment compound) by weight (of potato tubers) is shown in Table 25.

**Table 25. CIPC trial quantities.**

| Date of Application | CIPC (Wet Spray) | HPβCD | CIPC x HPβCD |
|---|---|---|---|
| 13.11.14 | 12 g/tonne | 78.4 g/tonne | 12 g/tonne CIPC + 78.4 g/tonne HPβCD |
| 04.02.15 | 12 g/tonne | 78.4 g/tonne | 12 g/tonne CIPC + 78.4 g/tonne HPβCD |
| 24.03.15 | 12 g/tonne | 78.4 g/tonne | 12 g/tonne CIPC + 78.4 g/tonne HPβCD |
| 13.05.15 | 12 g/tonne | 78.4 g/tonne | 12 g/tonne CIPC + 78.4 g/tonne HPβCD |

*S*-Carvone was complexed with βCD, and equal quantities of both *S*-carvone and βCD alone were also used as treatments. Sufficient treatment compound was prepared for 45 Kg of potato tuber in 2 L water. *S*-Carvone alone and *S*-Carvone complexed with βCD were also prepared with 1% ethanol in order to improve the solubility of *S*-Carvone in solution. Therefore, a 1% ethanol control was also included in this trial. Samples were prepared 24 hours prior to treatment and were left at room temperature overnight in order to allow complexation between *S*-Carvone and βCD to occur. The quantity used to treat each sample was calculated relative to potato tray weight. Volume (of treatment compound) by weight (of potato tubers) is shown in Table 26.

**Table 26. *S*-Carvone application quantities.**

| **Date of Application** | ***S*-Carvone (+ 1% ethanol)** | **βCD** | ***S*-Carvone x βCD (+ 1% ethanol)** | **1% ethanol** |
|---|---|---|---|---|
| 13.11.14 | 111.6 ml/tonne (+ 1% ethanol) | 269.8 g/tonne | 111.6 ml/tonne *S*-Carvone + 269.8 g/tonne βCD (+ 1% ethanol) | 10 ml/L |
| 16.01.15 | 111.6 ml/tonne(+ 1% ethanol) | 269.8 g/tonne | 111.6 ml/tonne *S*-Carvone + 269.8 g/tonne βCD (+ 1% ethanol) | 10 ml/L |
| 18.03.15 | 111.6 ml/tonne(+ 1% ethanol) | 269.8 g/tonne | 111.6 ml/tonne *S*-Carvone + 269.8 g/tonne βCD (+ 1% ethanol) | 10 ml/L |
| 13.05.15 | 111.6 ml/tonne(+ 1% ethanol) | 269.8 g/tonne | 111.6 ml/tonne *S*-Carvone + 269.8 g/tonne βCD (+ 1% ethanol) | 10 ml/L |

Proportion of tubers showing signs of sprout development ('eyes open') was recorded prior to all treatments being applied (Figure 4). Average sprout number and length were recorded each week over a 9 month period when visible sprouts began to develop. Sprout length was recorded as longest sprout per tuber. Average length of sprout was recorded when greater than 1 mm. Below 1 mm, sprout length was recorded as < 1 mm. Average sprout length has been analysed when sprouts were recorded as greater than 1 mm in length. A sample size of 10 tubers per treatment was used for this observational analysis.

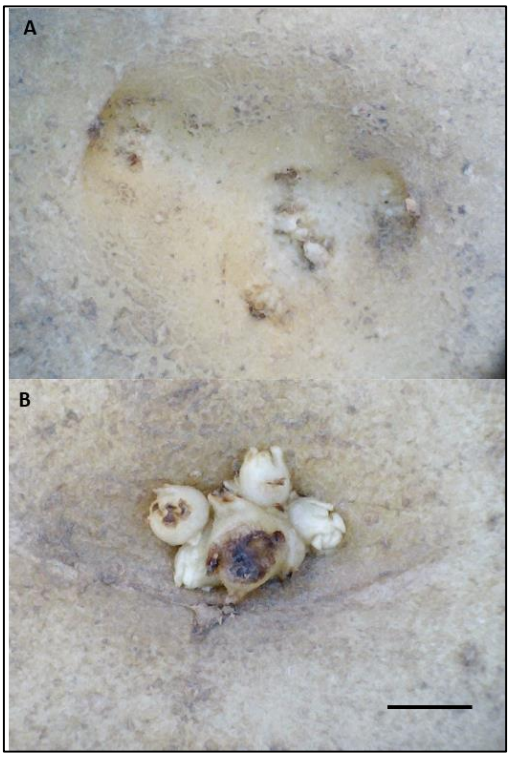


**Figure 4. Potato sprouts.**

A. 'Eyes open'; B. Sprout.
Scale bar = 5 mm.

### 2.2.4 Statistical Analysis

Non-parametric Kruskal-Wallis testing and Mann Whitney U post-hoc analysis was used to test for significant differences in the data sets. Non-parametric tests use rank data to test for significance, and therefore the null hypothesis states that two compared data sets come from the same population. GenStat (Version 16) was used to carry out this statistical analysis.

### 2.2.5 Fry Colour Analysis

Fry samples were produced at reglar intervals throughout the trial. A sample of 10 potato tubers was used for crisp production by the Nelson County Potatoes Quality Control Team to determine the quality of crisps produced from potato tubers treated in this trial. This quality control process involved slicing and frying 300 g of potato tuber to check fry colour. Once fried, crisps were compared to the Potato Council Fry Colour Chart (Appendix 3) to ensure that they would be of a suitable colour quality for the consumer. 50 mL of homogenised potato tuber tissue was also used for sugar analysis using the YSI 2950 Biochemistry Analyser (YSI Life Sciences). Sucrose and glucose concentrations of the potato tuber samples were assessed using this machine in order to ensure that they were within the bounds set by the consumer (data not shown).

### 2.2.6 Residue Extraction and Analysis

Methanol was used as a solvent in order to extract CIPC and *S*-Carvone from potato tuber skin samples (varieties: Markies and Hermes). 1 g of fresh weight tuber skin sample was removed from potatoes treated with each of the following compounds: Untreated, CIPC (Hot fog), CIPC (aqueous spray), *S*-Carvone. Samples were placed in (10 ml) vials in 8 ml methanol, sonicated for 5 minutes in a sonication bath and then left for one week at room temperature (22 °C) in order for compounds to be extracted into the solvent.

### 2.2.7 LC-UV method

The Shimadzu Single Quad Instrument (Metabolomics Department, John Innes Centre) was used for Liquid Chromatography – UV (LC–UV) analysis of methanol extracts. 0.1% formic acid (Solvent A) and acetonitrile (Solvent B) were used. The Luna 3u C18 (100 mm x 2.0 mm) column was used for liquid chromatography. Injection volume used was 5 µl, and flow rate of 0.4 ml/min. Detection method used PDA, scanning wavelengths from 200-500 nm. The following programme was used.

**Table 27. LC-UV method.**

| Time | Solvent |
|---|---|
| 0 – 5 minutes | 50 % B |
| 5 – 7 minutes | 90 % B |
| 7 – 9 minutes | 90 % B |
| 9 – 10 minutes | 50 % B |
| 10 – 13 minutes | 50 % B |

Chemical standards of CIPC and *S*-Carvone were used in order to produce a calibration curve which could be used to approximate concentrations of these compounds in samples. Concentrations of 0.1, 0.01 and 0.001 mg/ml were used for each standard.

### 2.2.8 *S*-Carvone Sealed Box Trial

#### 2.2.8.1 Trial Set-up and Observational Sprouting Analysis

A sealed box trial was set up in which the effect of atmospheric *S*-Carvone on potato sprouting was assessed. Potato tubers (variety: Markies) were removed from the mass storage facility following the third hot fog CIPC application (14.03.15). 20 tubers were placed into each transparent box (H: 25.4 cm, L: 43.6 cm, W: 33.9 cm) and an *S*-Carvone dilution series was used in each of the 10 boxes. *S*-Carvone concentrations shown in Table 28.

**Table 28. *S*-Carvone box trial quantities.**

| | Box 1 | Box 2 | Box 3 | Box 4 | Box 5 |
|---|---|---|---|---|---|
| Farm stored tubers (9 °C) | 0 | 0.058 ml/kg | 0.116 ml/kg | 0.224 ml/kg | 0.335 ml/kg |
| Lab stored tubers (22 °C) | 0 | 0.058 ml/kg | 0.116 ml/kg | 0.224 ml/kg | 0.335 ml/kg |

*S*-Carvone was placed in unsealed 10 ml glass vials and were attached to the box in order to allow for the volatile *S*-Carvone to circulate within the atmosphere of the sealed box. All boxes were stored in the dark for a period of 8 weeks. 5 boxes were stored at 9 °C in the cold box storage facility at Nelson County Farm and 5 boxes were stored at room temperature (22 °C) at the John Innes Centre (Figure 5).


**Figure 5. Sealed box *S*-Carvone trial.**

### 2.2.8.2 Statistical Data Analysis

Average sprout number and average sprout length were recorded before potato tubers were placed into sealed transparent boxes, and were also recorded once the boxes were opened at the end of the trial. Sprout length was recorded as longest sprout per tuber. Photographs were taken of the boxes on a weekly basis to document the development of sprouting throughout the trial. Kruskal-Wallis non-parametric testing and Mann-Whitney U post-hoc tests were used to analyse statistical significance in the data sets. This was carried out using GenStat 16 software.

### 2.2.8.3 Residue Extraction and Analysis

A Solid Phase Micro Extraction (SPME) procedure was used to determine the presence of *S*-Carvone in the potato sprouts. Potato tuber sprouts were removed from the tubers immediately upon opening the boxes and were frozen at -20 °C to prevent loss of volatile compounds. Head-space gas testing was used to determine the presence of *S*-Carvone in the sprouts. 3 g of fresh weight tuber sprouts were placed into a sealed 250 ml round-bottom flask which was then pierced by the SPME system and the fibre (fused silica/SS – 65 µm PDMS/DVB*)* was exposed to headspace gases for 5 minutes to allow for extraction of headspace gas compounds (Figure 6).

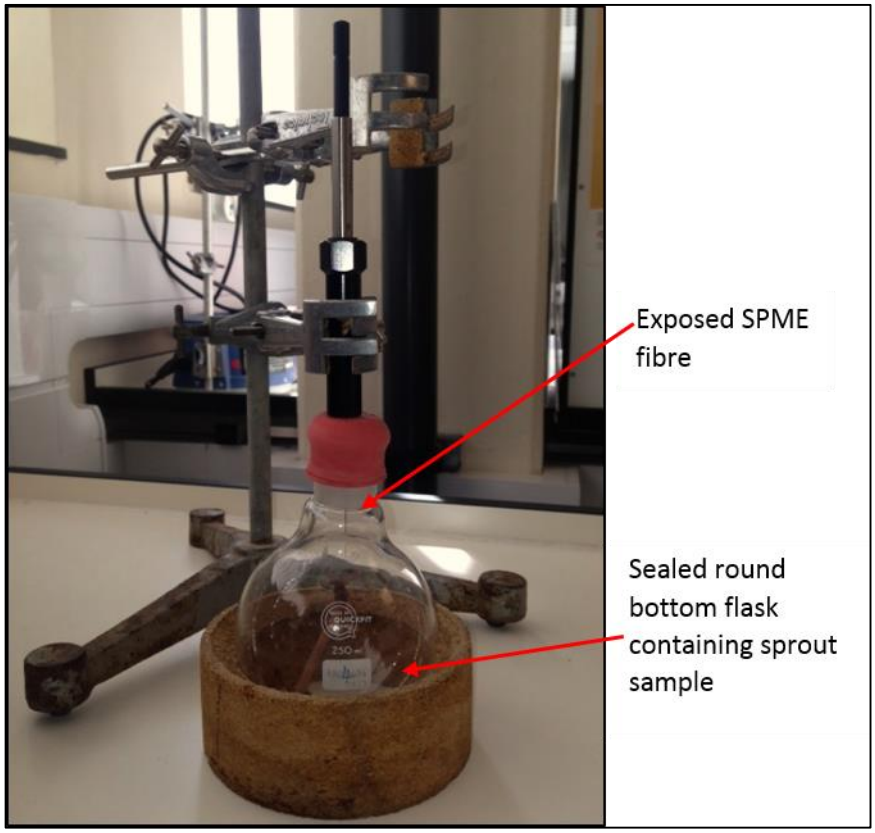


**Figure 6. SPME-Headspace gas analysis of potato sprout samples.**

The SPME fibre was subsequently transferred to the injection port of the GC-MS to allow desorption of the compounds. The extracted compounds were analysed by GC-MS. GC-MS measurements were made using an Agilent Technologies 6890N network GC system. The column used for analysis was a ZB-5HT column (30 m + 5 m guard column, 0.1 µm film thickness). Mass spectra and Total Ion Current (TIC) chromatograms were obtained using scanning in the mass range m/z 45-300. Extracted Ion chromatograms are shown in results section for clarity.

**Table 29. GC-MS temperature cycle programme for *S*-Carvone detection.**

| **Temperature cycle** | **Temperature** | **Time** |
|---|---|---|
| Injection Temperature | 250 $^{o}$C | - |
| Initial Oven Temperature | 50 $^{o}$C | 5 minutes |
| Ramp 1 | 50 $^{o}$C – 150 $^{o}$C<br>150 $^{o}$C | 20 $^{o}$C/minute<br>Hold 5 minutes |

Ionisation method for mass spectrometry used electron ionisation (EI) at 70 eV.

# 3 Solutions for the Potato Cyst Nematode as a Pathogen of the Potato Crop

## 3.1 Introduction

This chapter will look at the Potato Cyst Nematode as a pathogen of the potato crop, and provides a preliminary study for attempting to find a solution to this problem.

### 3.1.1 Evolution of the Potato Cyst Nematode

Potato Cyst Nematode (PCN) (*Globodera rostochiensis, Globodera pallida*) is a major plant pathogen, which infects species of the genus *Solanum*, with potato (*Solanum tuberosum*) being the major host. The tetraploid potato evolved in the Andes as a result of hybridisation between two wild diploid Solanum species: *S. stenotomum* and *S. sparsipilum* around 10,000 years ago (Hawkes 1988). PCN is believed to have co-evolved in the Andes along with its predominant host, the potato (Canto Saenz and De Scurrah 1977).

The idea of plant-pathogen co-evolution is well established. Plant populations are often polymorphic for pathogen resistance. However, pathogens also tend to be polymorphic for virulence genes which overcome the resistance of plants. Plants are at a selective advantage if they are able to resist a specific mechanism of attack by a pathogen. In response to this, pathogens, which are able to overcome such plant resistance have a selective advantage. By this model, plant and pathogen populations continually co-evolve (Frank 1992). This relationship is often termed an 'evolutionary arms race'. In the example of the potato and PCN, nematodes are likely to have evolved virulence against the resistance genes of the potato.

With the domestication of the potato and the industrialisation of agriculture, PCN has now spread to temperature regions globally, and is thought to infest potato crops in 65 countries (Warner 2008). Picard *et al. (*2004) highlighted extensive gene flow within regions of up to 35 km. It was suggested that passive dispersal of cysts by natural means including wind, water or by wild animals, along with anthropogenic means, such as the movement of infected seed tubers, may explain the level of gene flow (Picard, Plantard et al. 2004). The movement of agricultural machinery and harvested crops has also aided the spread of PCN and now mean that it is a global problem for potato crops. The fact that cysts can be moved easily over a wide area explains why quarantine programs are no longer successful in limiting the spread of the nematode. Furthermore, the long life cycle and robust physiology of PCN mean that cysts may remain dormant for long periods of time before infecting a host.

It is unclear how PCN was originally spread from South America to Europe, however Evans *et al.* (1975) suggested that PCN may have been brought to Europe following the importation of tuber collections from South America. These collections were used for breeding varieties with resistance to Potato Blight (*Phytophthora infestans*) in 1845-1846. Whilst this breeding improved resistance to blight, it is also likely to have resulted in the establishment of PCN in Europe. Despite this, PCN was first documented in Europe, in Germany, in 1881: 30 to 40 years after it is thought to have been introduced. During this time, field population levels had the chance to become established and build up to noticeable levels (Evans, Franco et al. 1975) .

### 3.1.2 Potato Cyst Nematode Life Cycle

*Globodera rostochiensis* and *Globodera pallida* can be distinguished by the differences in the colours of the cuticle of mature females at the onset of cyst development (Wouts 1976). Second-stage juvenile (J2) eggs are contained within cysts, which hatch in response to a hatching factor present in 'Potato Root Diffusate' (Rawsthorne and Brodie 1987). J2 juveniles invade host plant roots and migrate intra-cellularly through root cortical cells towards the vascular cylinder. Upon reaching the vascular cylinder, juvenile cyst nematodes establish a feeding site (syncytia) by injecting stylet secretions (Hussey 1989). The formation of syncytia is due to fusion of root cells accompanied by cell wall degradation and protoplast fusion. Expansins, cell wall-loosening proteins, have been shown to be up-regulated in syncytia (Wieczorek, Golecki et al. 2006). After several days of feeding, J2 juveniles moult and progress through third-stage (J3) and fourth-stage (J4). At this stage male J4 juveniles cease feeding, whilst females continue to feed. After moulting to their adult stage, mating occurs, and eggs are produced (Golinowski, Sobczak et al. 1997). Eggs are stored within the female's body. The female dies and its body becomes the protective cyst (Williamson and Gleason 2003), which may lie dormant in the soil until stimulated to hatch by the presence of potato root diffusate . The cyst is spherical and is approximately 0.5 mm in diameter. Newly formed cysts usually contain 200-600 larvae, and cysts may remain dormant in the soil for over 10 years (Gratwick 1992) (Figure 7).

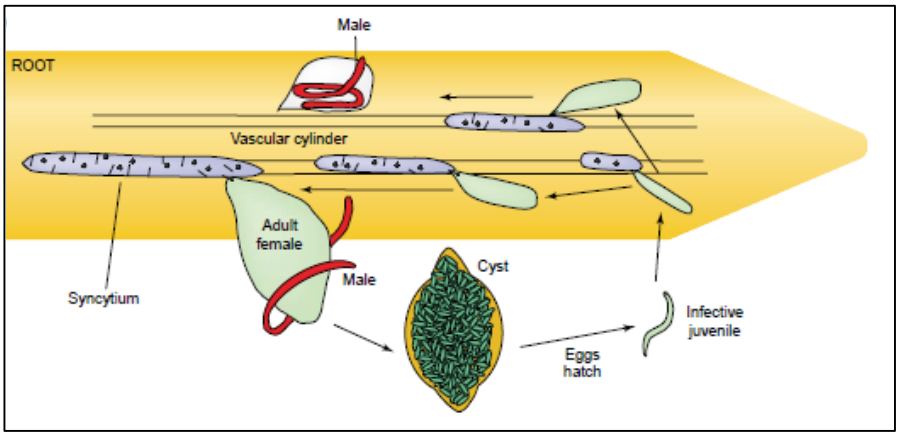


**Figure 7. Life cycle of Potato Cyst Nematode.**

Reproduced from Williamson & Gleason (2003) (Williamson and Gleason 2003), with permissions from Elsevier.

Features of the nematodes life cycle and morphology provide explanations for the success of the pathogen. The robust cyst which holds eggs protects the juveniles from abiotic stresses, such as desiccation, and the high number of offspring produced per female results in rapid population growth.

### 3.1.3 Effect of PCN on the Potato Crop

PCN has a major impact on potato crop yield globally; a study carried out in 2002 by Minnis et al. suggested that 64% of sampled potato fields in England and Wales were infested with PCN, the majority of which were with *G. pallida* (Minnis, Haydock et al. 2002). However, current estimates suggest that PCN affects approximately 80% of potato crops in the UK (personal communication: (Tomalin 2014)), and therefore is a major threat to the UK potato market. PCN infected crops display symptoms similar to those of plants with inefficient rooting systems, such as poor growth, wilting and early senescence. This is due to *G. rostochiensis* and *G. pallida* juveniles feeding on the root vasculature, therefore limiting the nutrient supply for plant growth. Plants infected with PCN generally have reduced nitrogen, phosphorus and potassium concentrations, and a study by Ruijter & Haverkort (1999) suggested that nematodes reduce nutrient uptake by the plant (De Ruijter and Haverkort 1999).

Clearly, the potato industry loses a great deal of its yield due to the fact that 80% of UK potato fields are infested with PCN. Whilst PCN tends not to be lethal to plants, it does significantly stunt growth, and therefore the crop is not as productive as it would be without the presence

of the pathogen (Figure 8). Methods of controlling PCN are crucial for maximising potato yield in the future.

**Figure 8. PCN infected potato field.**

### 3.1.4 Conventional Treatment Methods

Several methods are used by farmers at present to limit the damage caused by PCN to potato crops and these include the use of both nematicides and trap crops. However, both methods have their disadvantages.

Resistant potato cultivars are used to limit the effect of PCN on the crop, however, this is a limited solution. The H1-resistant potato cultivar, resistant against *G. rostochiensis* was identified by Ellenby (1954) (Ellenby 1954). However, the use of this resistance gene for mitigating the effects of *G. rostochiensis* infection has led to a drastic increase in the proportion of *G. pallida*. Furthermore, there are no potato cultivars available on the UK National List with full resistance to European *G. pallida* pathotypes, and therefore the use of partially resistant cultivars also requires the use of nematicides to limit the damaging effects of PCN (Turner, Martin et al. 2006).

Although nematicides can protect potato crops against PCN, this is a costly method of mitigation. In 2008, the Potato Council reported that approximately 23% of the total UK potato area was treated for PCN and/or free living nematodes, at a cost of around £9 million (Council 2008). Furthermore, as has been discussed elsewhere in this thesis, changes to EU regulations result in the withdrawal of pesticides and other agrochemicals from the industry. Reliance on chemical

control of a pathogen as virulent as PCN may ultimately result in a total loss of control of the pathogen.

The use of crop rotation to allow PCN populations in the soil to decline naturally has been suggested. This includes trap cropping, which involves growing a crop specifically to induce PCN egg hatching. Once the eggs hatch, there is no host present for the larvae to infect and therefore the larvae die. *Solanum sisymbriifolium* (Sticky Nightshade) is not a host plant for PCN, but has been shown to induce a high level of nematode hatching before leaving the juveniles to die (Scholte and Vos 2000); (Dias, Conceição et al. 2012)). Based on these results, *S. sisymbriifolium* could be considered a useful trap crop, as it promotes nematode hatch, but does not facilitate reproduction of the nematode as the plant is resistant. Indeed, this method of biological control may be attractive as it is unlikely to be subject to legislation changes. However, due to the morphology of the cyst and its ability to persist for several decades in the soil before hatching, this is an uneconomical suggestion. Following personal communication with farmers, it seems unrealistic to expect farmers to use this method of crop rotation to control PCN, due to the fact that *S. sisymbriifolium* is a difficult crop to germinate. Furthermore, it is uneconomical for farmers to grow a crop that will not directly contribute to their profits.

Clearly, the current methods of control for PCN are sub-optimal. Methods of control in the future would ideally prevent hatching of PCN, and therefore research has been carried out to fully understand the mechanism by which nematode hatching is stimulated.

### 3.1.5 Hatching Factors

Work to understand the relationship between the potato and the nematode has led to interesting findings. A secondary metabolite produced by the plant, known as Solanoeclepin A, has been isolated as a 'hatching factor' in response to which nematodes will hatch from their cysts (Mulder, Diepenhorst et al. 1996). Solanoeclepin A therefore may be the active component present in the 'Potato Root Diffusate' described by Rawsthorne and Brodie (Rawsthorne and Brodie 1987).

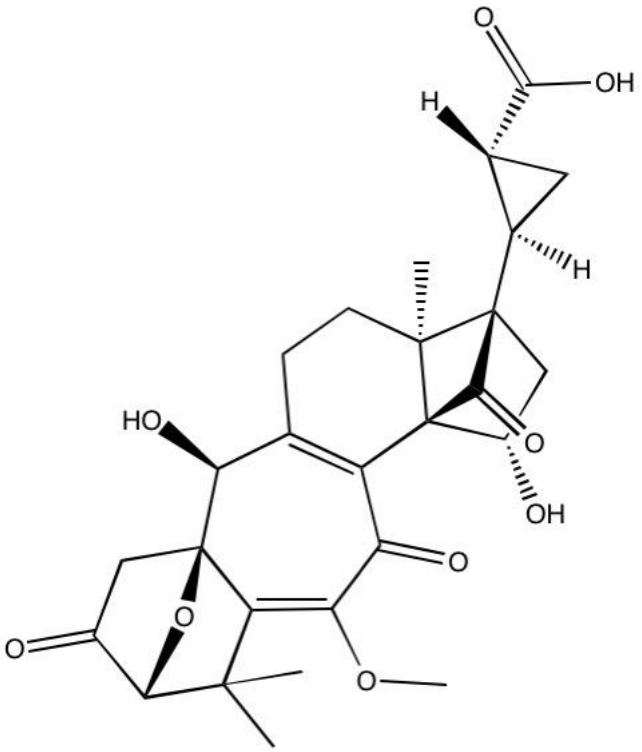


**Figure 9. Solanoeclepin A structure.**

Through the release of Solanoeclepin A, the potato stimulates nematode hatch, and ultimately the infection of the plant. This concept underlies many plant-pathogen relationships, and it is well established that pathogens respond to chemical compounds released by a plant as a stress response. The pathogen uses this as a cue that the stressed plant is in the vicinity and can then attack the plant. Examples of this concept have been demonstrated in other plant-nematode interactions. For example, plants under insect attack have been shown to release volatiles in large quantities, and hence this signal may serve as a cue for nematodes to locate an environment with a potential host (Turlings et al. 2012). Knowledge of the hatching factor in the Potato Cyst Nematode case is therefore critical for finding a solution to minimise the effect of PCN on potato crops.

The heptacyclic structure of Solanoeclepin A was elucidated by Schenk *et al.* (1999) (Schenk, Driessen et al. 1999). However, this structure does not appear to have been confirmed in other subsequent studies. Solanoeclepin A ($C_{27}H_{30}O_9$) is believed to be a triterpenoid (derived from a triterpene), and has a highly complex structure.

The structure of Solanoeclepin A is unusual in the fact that it contains three, four, five, six and seven-membered carbon rings. Whilst the chemical synthesis has been achieved by Tanino *et*

*al.* (2011) (Tanino, Takahashi et al. 2011), this process took 10 years and required 36 synthetic steps. Clearly, chemical synthesis of Solanoeclepin A for use as a crop treatment to promote premature nematode hatching is not feasible. However, elucidating the biosynthesis of Solanoeclepin A synthesis may provide useful insight, which can ultimately be used for producing nematode resistant varieties.

### 3.1.6 Triterpenoid Synthesis

Triterpene saponins have been shown to play a role in protecting against pathogens and pests (Osbourn, Goss et al. 2011), therefore Solanoeclepin A may be the subject of an evolutionary arms race, during which nematodes hatch in response to a compound that is released to protect the plant from other pathogens. This hypothesis supports the idea of the potato and PCN co-evolving.

The triterpenes are 30-carbon structures derived from oxidosqualene (Chappell 2002) and are a numerous and diverse group of plant natural products. However, they are also highly complex, and largely cannot be produced through chemical synthesis (Thimmappa, Geisler et al. 2014). This explains the fact that many steps and a long time period were required for the chemical synthesis of Solanoeclepin A by Tanino *et al.* (2011). Whilst the biosynthetic pathways of specific triterpenes and triterpenoids, such as Solanoeclepin A, are unknown, they share a well-characterised common biosynthetic origin, known as the mevalonate pathway (Chappell 2002). The final step of this pathway is the cyclisation of 2,3-oxidosqualene by oxidosqualene cyclases (OSCs). The cyclisation product of OSCs is the first step in the diversification process of triterpenes, as OSCs produce a variety of products with different stereochemistry, conformations and ring numbers (Thimmappa, Geisler et al. 2014). This knowledge, along with the potato genome sequence, may be used to predict potential biosynthetic pathways of Solanoeclepin A.

An investigation into terpene diversification across multiple sequenced plant genomes has identified terpenoid synthase 'signature' enzymes (TS), responsible for generating scaffold diversity. Furthermore, cytochromes P450 enzymes (CYPs), involved in modifying and further diversifying the scaffold have also been identified as major enzymes involved in terpene diversification (Boutanaev, Moses et al. 2015). Interestingly, they found that TSs and CYPs pairs are often found together and certain gene pairs predominate, suggesting that such genes may be key in the terpene diversification process. This is consistent with the fact that some terpene

biosynthesis pathways in *Solanum* species have been shown to be clustered, for example genes for the biosynthesis of α-tomatine in the tomato (Nutzmann and Osbourn 2014). The identification of novel gene clusters in plant genomes could be exploited for elucidation of alternative terpene biosynthesis pathways.

Similar work to identify triterpene biosynthetic pathways has been carried out in oats (*Avena* spp.). Avenacins, synthesised from a triterpene known as β-amyrin, are antifungal compounds, which provide protection against disease. The first committed enzyme in the avenacin biosynthetic pathway has been found to be the β-amyrin synthase gene *AsbAS1* (Haralampidis, Bryan et al. 2001). This has been shown to be clustered with other genes required for avenacin biosynthesis (Qi, Bakht et al. 2004). Subsequent work has identified a gene encoding a member of the CYP51 family of cytochrome, which further modifies the triterpene scaffold (Geisler, Hughes et al. 2013). Studies showing parallels with the elucidation of Solanoeclepin A biosynthesis will be useful for identifying candidate enzymes involved in the pathway.

### 3.1.7 Hypothesised Enzymatic Synthesis of Solanoeclepin A

A potential method of mitigating the harmful effects of Potato Cyst Nematode (PCN) infection on potato crops may involve either an application of the hatching factor compound prior to crop planting to force premature nematode hatch, or to genetically engineer a potato variety that does not synthesise this compound, and therefore doesn't signal to nematodes to hatch. Regardless of the options for mitigating the effects of the nematode on the potato crop, knowledge of the biosynthesis of this compound is interesting and useful in order to further understand the interaction between pathogen and host.
The complex structure of Solanoeclepin A (chemical formula: $C_{27}H_{30}O_9$) implies that there are various possibilities for the native biosynthetic pathway in potato.

Solanoeclepin A has been referred to as a 'unique triterpenoid with a hitherto unknown heptacyclic skeleton containing carbocycles with each of the ring sizes from three to seven' (Tanino *et al.* 2011). The unique structure of the compound has provided useful starting points for the hypothesis of a biosynthesis scheme, due to the rarity of several aspects of the structure, such as the presence of a cyclopropane ring and the ring expansion from a 6-carbon membered ring to a 7-carbon membered ring. It should also be noted that triterpenoids are derivatives of triterpenes, which have a chemical formula of $C_{30}H_{50}$, and therefore major modification of the triterpene from which Solanoeclepin A is derived must occur in order to reach a product with the formula: $C_{27}H_{30}O_9$.

If Solanoeclepin A is derived from a triterpene, an oxidosqualene cyclase (OSC) must be involved in the cyclisation of 2,3-oxidosqualene. OSCs from a variety of plants have been well characterised and reviewed by Osbourn *et al.* amongst others and have been shown to be involved in the production of triterpenes. Due to the structure of Solanoeclepin A, cycloartenol, or a compound with a similar structure, may be a candidate precursor for the biosynthesis of Solanoeclepin A. Cycloartenol is the compound from which plants, algae and some protists synthesise sterols, in contrast with lanosterol, which animals and fungi use for sterol synthesis (Gas-Pascual, Berna et al. 2014). Plants may then recruit these sterols into their secondary metabolic pathways, as is seen during steroidal glycoalkaloid biosynthesis (Itkin, Heinig et al. 2013).

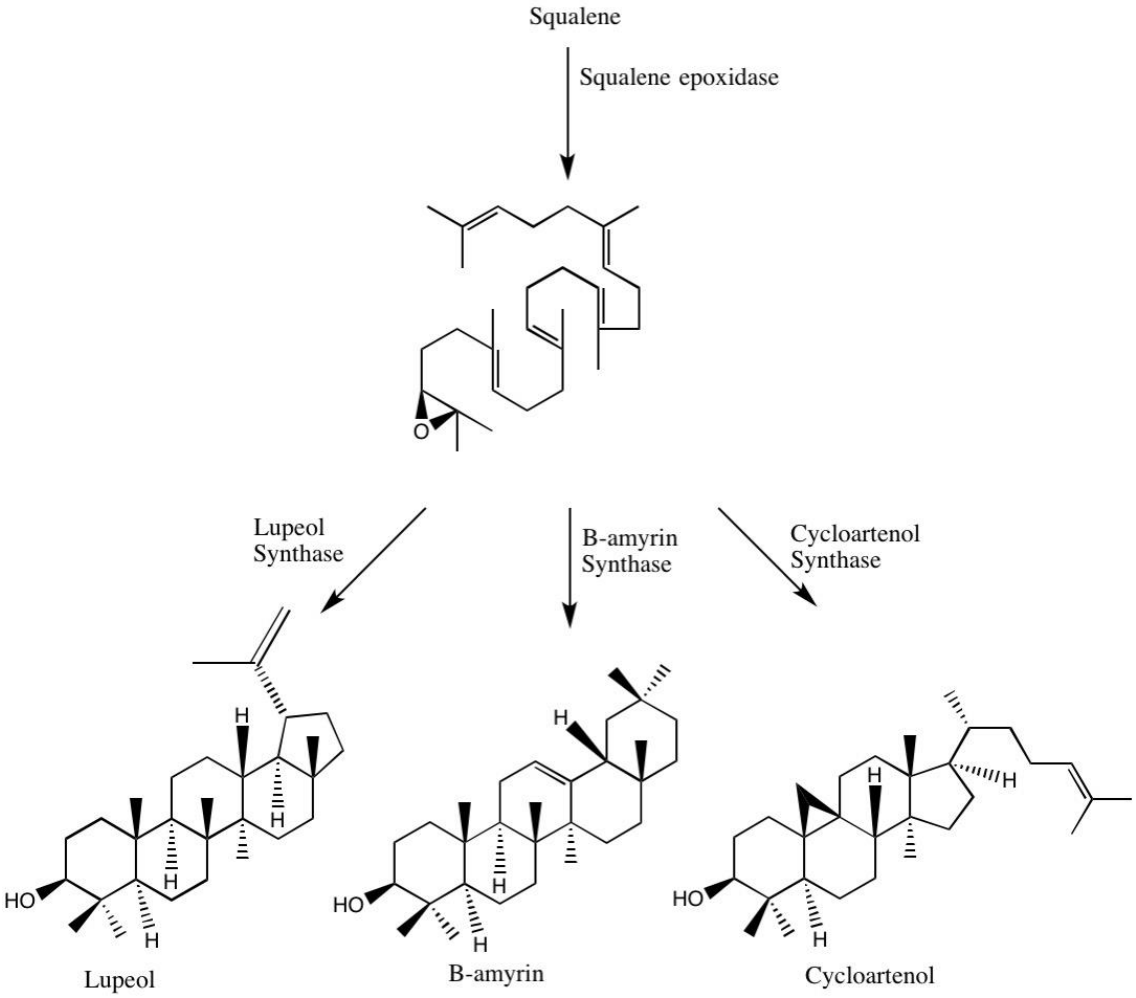


**Figure 10. Triterpene biosynthesis from squalene.**

Through the action of squalene epoxidase and oxidosqualene cyclases (OSCs).

It is clear that four carbon atoms need to be removed from the standard triterpene structure; and a methyl group adjoining the cycloheptane ring must be added during a downstream modification step, to result in a compound with a $C_{27}$ structure. Along with the loss of four carbon atoms, a carboxyl group must be acquired. Therefore a C-C cleavage reaction is likely to occur. An example of this is seen in the biosynthesis of pregnenolone from cholesterol (Mast, Annalora et al. 2011).

In order to form the final $C_{27}$ structure, a methyl-transferase is likely to add a methyl group to the cycloheptane ring, and therefore add the final carbon to this structure. This step is likely to be a downstream modification of the triterpenoid backbone structure.

Following the formation of the $C_{27}$ backbone structure, several further major modifications are required for the production of Solanoeclepin A. Ring expansion of a six-carbon membered ring to a seven-carbon membered ring may be feasible due to the presence of the cyclopropane ring in cycloartenol (Figure 11). The cyclopropane ring of cycloartenol (Figure 11, Carbon no. 19) may be involved in the ring expansion step which leads to the cycloheptane ring present in Solanoeclepin A. The fact that cycloartenol has the cyclopropane ring suggests that it is a more likely candidate for Solanoeclepin A biosynthesis compared to lanosterol, although the evidence for this hypothesis is lacking in the literature.

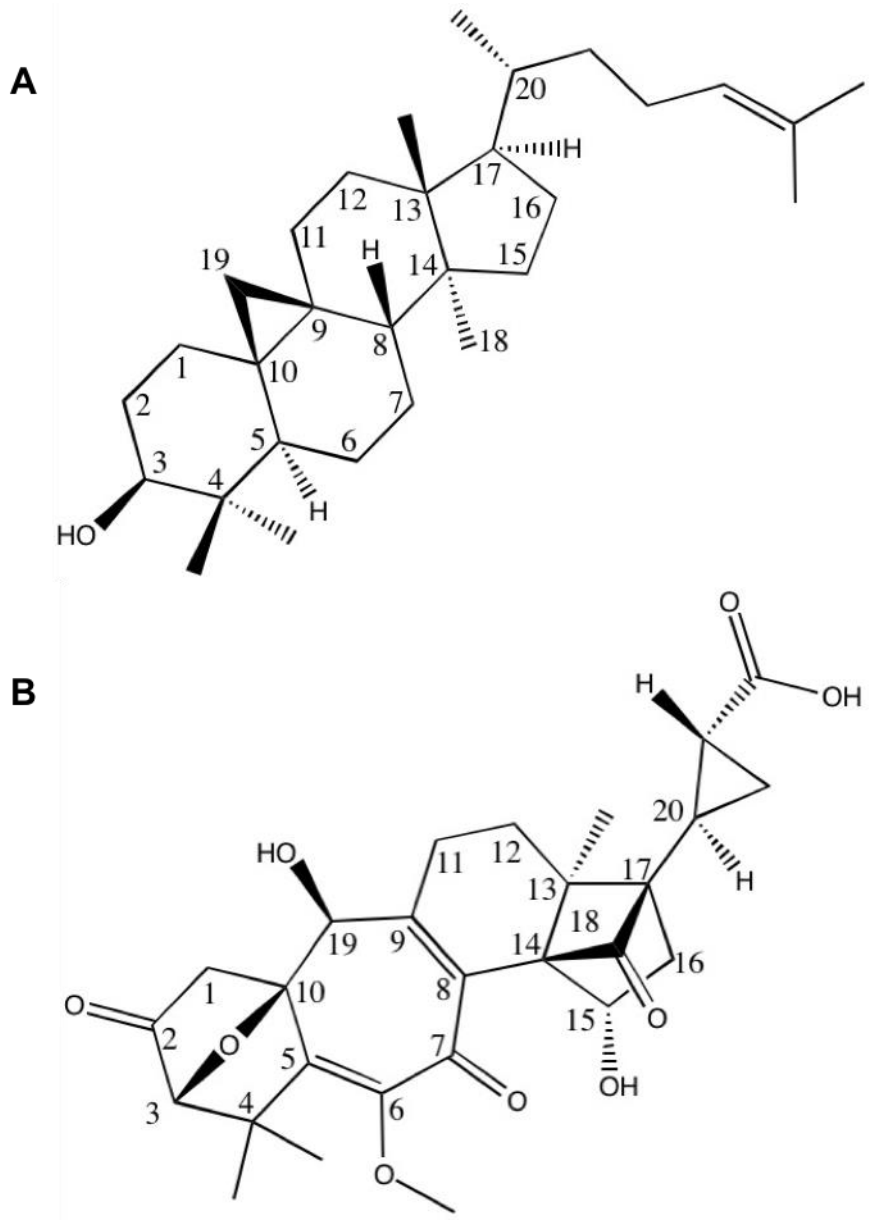


**Figure 11. Comparison of Cycloartenol and Solanoeclepin A structures.**

A) Cycloartenol, (B) Solanoeclepin A.
Numbers display the hypothesised location of carbon atoms in Solanoeclepin A compared to cycloartenol. Numbering of Solanoeclepin A structure taken from Schenk *et al.* 1999; cycloartenol numbering follows Solanoeclepin A numbering.

The cyclobutane structure present in Solanoeclepin A (Figure 11, Carbon no. 18) is of interest as the cyclobutane ring is highly strained, and therefore rarely occurs in biological systems. The elucidation of the biosynthesis of this component of Solanoeclepin A will be interesting as this is not well documented in the literature. However, the structure of cycloartenol displays stereochemistry around carbon 13 and 14 (Figure 11) that may significantly affect the formation of the cyclobutane ring.

The formation of the cyclopropane ring is also of interest for the biosynthesis of Solanoeclepin A (Figure 11, Carbon no. 20). Whilst there are clearly cyclopropane rings in various natural products, including with the side chains of sterols (various mechanisms shown in review by Wessjohann *et al*. 2002(Wessjohann, Brandt et al. 2003)), there appears to be no literature at present identifying a cyclopropane ring in the same position as that seen in Solaneclepin A.

Further downstream modifications may involve the activity of monooxygenase cytochromes P450 for the addition of hydroxyl and ketone groups. The P450 families show huge diversity, and are responsible for catalysing a wide variety of monoxygenation reactions in primary and secondary metabolism in plants. Mizutani (2012) (Mizutani 2012) highlighted the fact that the diversity of saponins – a group of glycosylated triterpenes - in plants is dependent on the mode of cyclisation of 2,3-oxidosqualene and subsequent modification of the triterpenoid rings by oxygenation and glycosylation. Examples of such P450 modification are discussed by Seki *et al*. 2015, and are key to generating the structural diversity that is observed in triterpenes (Seki, Tamura et al. 2015). Therefore, it is likely that an array of cytochromes P450 are involved in the modification of the triterpenoid backbone structure of Solanoeclepin A.

Several mechanisms by which Solanoeclepin A may be synthesised by the plant have been described. However, the complexity of the compound in question means that there are likely to be an array of mechanisms by which Solanoeclepin A may be biosynthesised. For example, the fact that Solanoeclepin A is a $C_{27}$ compound immediately raises the question of whether the substrate is a $C_{25}$ or $C_{30}$ terpene. Whilst sesterterpenes (terpenes with a $C_{25}$ backbone) have been documented in the literature (Wang, Yang et al. 2013), they appear to be less prevalent than the $C_{30}$ terpenes, although this structure could also provide the backbone for Solanoeclepin A following significant modification.

In this study, OSCs have been selected as a starting point for the study of Solanoeclepin A in potato as they have been well characterised in other species. However, the diversity of triterpene structure means that products are difficult to predict from protein sequence alone, as OSCs may produce a variety of products with different functions.

### 3.1.8 Aims

It was hypothesised that a triterpene or sterol product is likely to be directly involved in Solanoeclepin A biosynthesis. Determining which product is involved requires the study of OSC

candidates. Whilst cycloartenol is a likely candidate for Solanoeclepin A biosynthesis, other OSCs should not be ruled out at this stage.

In order to investigate these hypotheses, I have attempted to characterise the triterpene metabolite profile of *N. benthamiana* overexpressing OSCs to determine the role that these enzymes play in Solanoeclepin A biosynthesis. OSCs make a huge array of diverse products; the specific OSC involved in triterpene and sterol product biosynthesis may not always be predicted from the OSC protein sequence. Therefore, a greater understanding of the function of potato OSC homologs will be crucial in elucidating the biosynthesis of a triterpenoid as highly complex as Solanoeclepin A. This requires the use of a heterologous expression system.

## 3.2 Results

### 3.2.1 Genome Analysis of OSCs in Potato

The potato genome (SpudDB) (Hirsch, Hamilton et al. 2014) and NCBI were used to identify OSC homologs present in potato. The SpudDB database identified several potato OSCs in duplicate, and many sequences identified were not full length genes. This meant that it was difficult to identify precisely how many putative OSCs are present in the potato genome. 13 OSCs have been identified in *Arabidopsis thaliana,* of which, two catalyse the biosynthesis of β-amyrin and one catalyses the biosynthesis of cycloartenol (Xue et al. 2011). Based on the structure of Solanoeclepin A, both β-amyrin synthase and cycloartenol synthase were identified as enzymes potentially involved in the synthesis of the end product as their products are structurally similar to Solanoeclepin A (as discussed in Section 3.1.7). Two putative β-amyrin synthases were identified from the NCBI database (XM_006364828 (2082 bp), XM_006351913 (2297 bp)) by BLASTing a characterised *Arabidopsis thaliana* β-amyrin synthase homolog (βAS) against the database. When these sequences were aligned against the *Solanum tuberosum* genome in the NCBI database, a putative delta-amyrin synthase was also identified (XM_006364827 (2487 bp)). A putative cycloartenol synthase was identified in the SpudDB potato genome database (PGSC0003DMT400073861) as well as in the NCBI database (XM_006340417 (2407 bp)) (Alignment in Appendix 1). These sequences are, as yet, putative as their functions have not yet been confirmed. This requires experimental confirmation. However, comparison between the cycloartenol synthase sequences from the two databases showed that the cycloartenol synthase candidate in the SpudDB database (PGSC0003DMT400073861) has a length of 1659 bp, as opposed to 2407 bp in the NCBI database. When these two sequences were aligned (Appendix 2), it was found that the SpudDB transcript was a truncated version of that identified in the NCBI database, suggesting that the SpudDB database may be misannotated. A list of candidate genes was narrowed down to those discussed, through the selection of only full length genes.

### 3.2.2 Phylogenetic Analysis of OSCs Across a Variety of Species

86 OSC sequences from a variety of species (Thimmappa, Geisler et al. 2014) were aligned using MEGA M6 and ClustalW software, and evolutionary relationships were determined using Maximum Likelihood phylogeny analysis (Figure 12). Several outgroup sequences were used in this analysis which included cycloartenol synthase homologs from *Chlamydomonas reinhardtii, Dictyostelium discoideum* and *Stigmatella aurantiaca.* Outgroup sequences for lanosterol synthases were from *Homo sapiens, Stigmatella aurantiaca* and *Methylococcus capsulatus.*

Outgroup sequences were used to root the tree, and were found on separate, longer branches of the tree to the main data set as they have come from organisms other than plants. There was lower sequence conservation between these outgroups compared to OSCs being analysed, and therefore they would be expected to be located further away on this tree. The phylogenetic tree generated from this data did not reflect species homology but clearly shows that the homologs of OSCs across species tended to cluster together by product rather than by species.

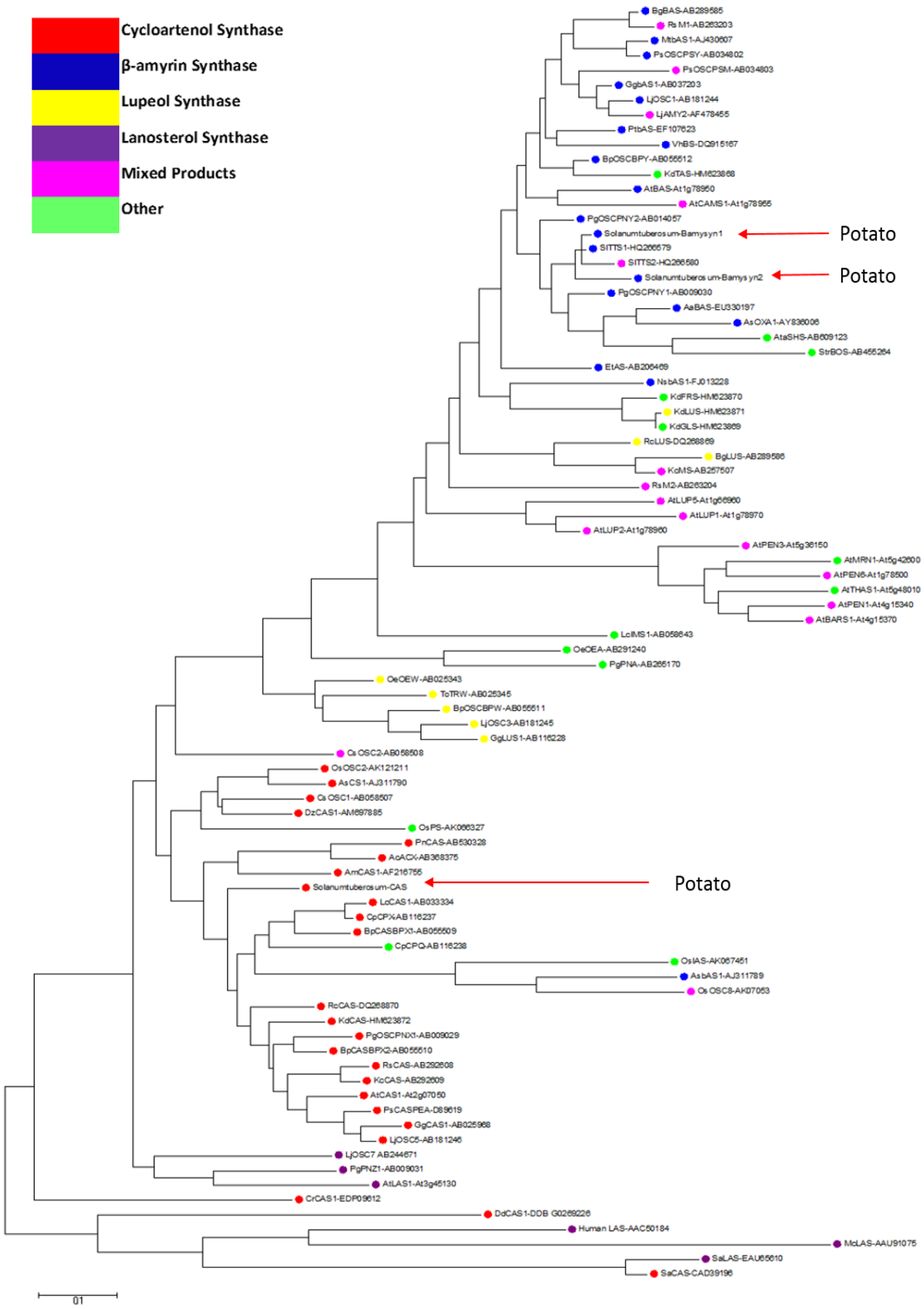


**Figure 12. Phylogenetic analysis of 86 OSCs from a variety of species.**

Maximum likelihood tree constructed in MEGA from 86 OSC protein sequences from a variety of species. Scale bar represents 0.1 amino acid substitutions per site. Colour key represents OSC product.

### 3.2.3 Differential Expression of OSCs in Potato Tissues

RNA was extracted from potato root, shoot and leaf tissue, and genomic DNA was also extracted. cDNA was reverse transcribed from extracted RNA samples. PCR was carried out using internal primers designed for various OSCs, and the different DNA samples as templates to determine differential expression of these genes in the potato. Expression profiling primers were used for this experiment; primers and expected size of amplicons are detailed in Materials & Methods (Section 2.1.1.4).

Differential expression of OSC genes was observed when PCR amplification was carried out using different cDNA templates from the potato. The predicted Delta-amyrin synthase was expressed in shoot and leaf tissue, but was not expressed in root tissue (Figure 13A). The predicted lupeol synthase was not expressed in root, shoot or leaf tissue, however was clearly present in the genomic DNA sample (Figure 13B). The predicted cycloartenol synthase and β-amyrin synthase were expressed in root, shoot and leaf tissue (Figure 13C, D). GAPDH (Figure 13E) was used as a control in this experiment, as this housekeeping gene should be expressed at equal levels in all tissue samples. The genomic DNA bands of interest observed in Figures (13B, C, and D) were larger than that observed for cDNA bands of the same gene as these primers were designed to span an exon-exon junction resulting in a larger DNA amplicon from the genomic DNA sample compared to the cDNA sample.

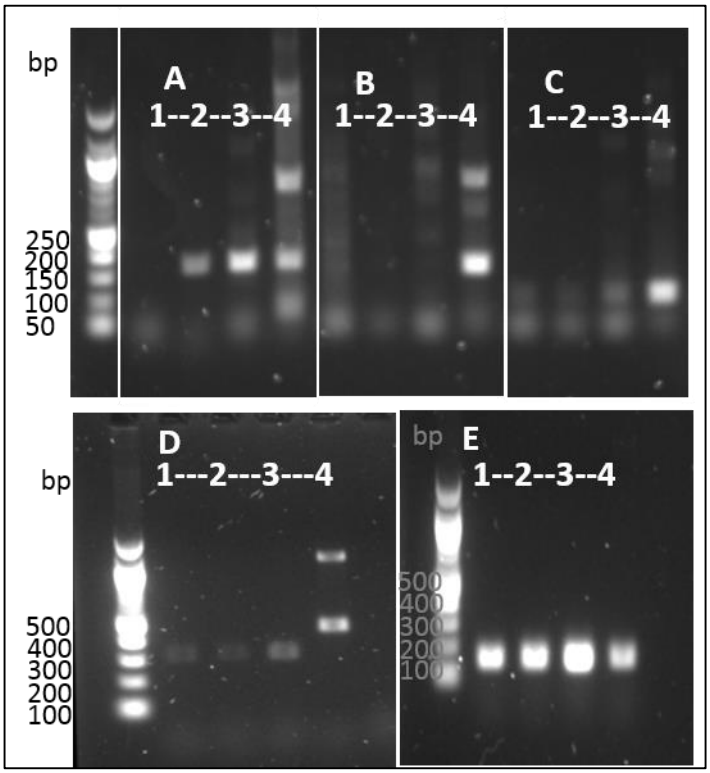


**Figure 13. Expression profiling.**

Agarose gels displaying expression profiling of various OSC genes using potato root, shoot, leaf RNA samples and genomic DNA for PCR. OSCs (A) Delta-amyrin synthase; (B) Lupeol synthase; (C) Cycloartenol synthase; (D) Beta-amyrin synthase; (E) GAPDH. GAPDH used as a control – this housekeeping gene should be expressed in all tissues. 4 wells for each gene on the gel representing different RNA/DNA samples. (1) Root RNA; (2) Shoot RNA; (3) Leaf RNA; (4) Genomic DNA. In this order across the 5 gels.

### 3.2.4 Optimisation of Cloning of Potato OSCs

Following the identification of OSC candidates in the potato genome, and determining their differential expression in tissue samples, candidates were cloned with the aim of transiently expressing the candidate genes in a heterologous host (*Nicotiana benthamiana*), in order to determine their function.

#### 3.2.4.1 Cloning of β-Amyrin Synthase

β-amyrin synthase (βAS) was cloned into the pEAQ-*HT*-DEST2 vector system (Sainsbury, Thuenemann et al. 2009) using Gateway cloning (Figure 14). β-amyrin synthase was amplified directly using potato leaf cDNA samples as templates, although the processes of PCR amplification and transformation into *E. coli* in the entry vector required significant optimisation.

Various PCR amplification protocols were used for this gene (Section 2.1.2.1.6), including both RT-PCR directly from RNA, and using cDNA synthesised in a separate reverse transcription step prior to high fidelity PCR. The Q5 High Fidelity Polymerase (NEB) was the most efficient method for amplifying this gene from cDNA. Following PCR, the TOPO cloning reaction was carried out and *E. coli* cells were transformed with the pCR8/GW entry vector carrying the β-amyrin synthase gene. Colony PCR was carried out to screen for colonies with the gene inserted in the correct orientation, as TOPO cloning may allow for the gene to be inserted in the reverse orientation. Approximately 50% of colonies had the gene inserted correctly (Figure 14C).

Interestingly, in the first instance of sequencing positive clones, all clones appeared to have a premature STOP codon inserted at base 1327 (Figure 15). This may be explained due to using a second PCR to amplify the product of an initial PCR reaction, in order to gain a higher concentration of DNA for the TOPO cloning reaction. If the polymorphism resulting in a premature STOP codon occurred during the early stages of the first PCR reaction, it is possible that all clones had this STOP mutation encoded as the mutation would be amplified a significant number of times following its occurrence. Therefore, PCR reactions were carried out again. Premature STOP mutations were not seen in the products of these reactions, however, it is clear that care must be taken when using PCR products as template for subsequent PCR reactions.

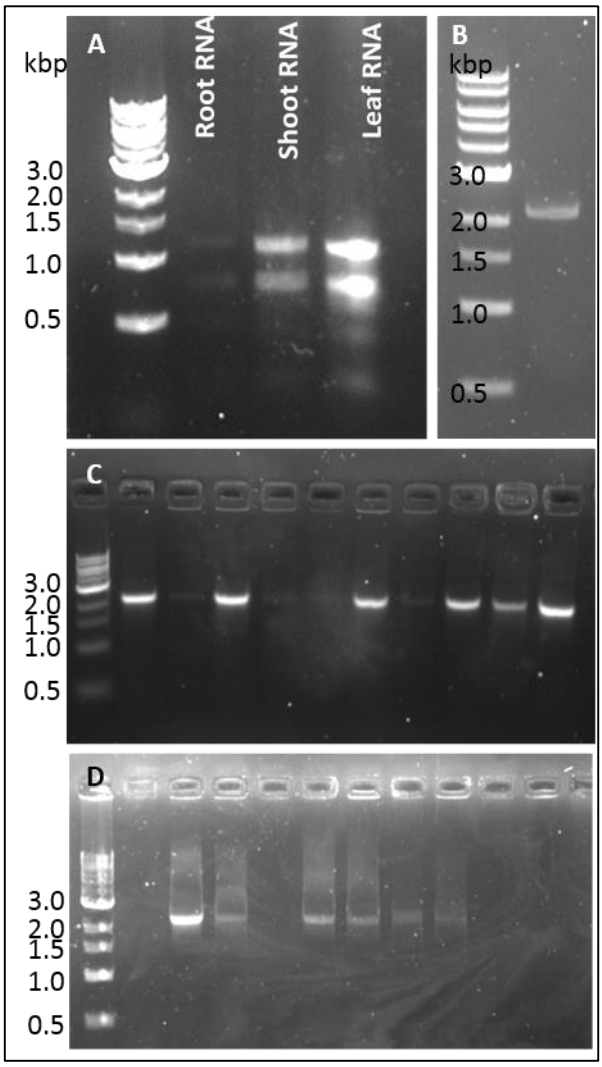


**Figure 14. Cloning of β-amyrin synthase (βAS).**

Agarose gels displaying different cloning stages of β-amyrin synthase (βAS). (A) RNA extracted from potato root, shoot and leaf samples; (B) β-amyrin synthase PCR product using Q5 Hi-Fidelity Polymerase; (C) β-amyrin synthase colony PCR following insertion in pCR8/GW vector; (D) β-amyrin synthase colony PCR following ligation using LR reaction into pEAQ-*HT*-DEST2 vector.

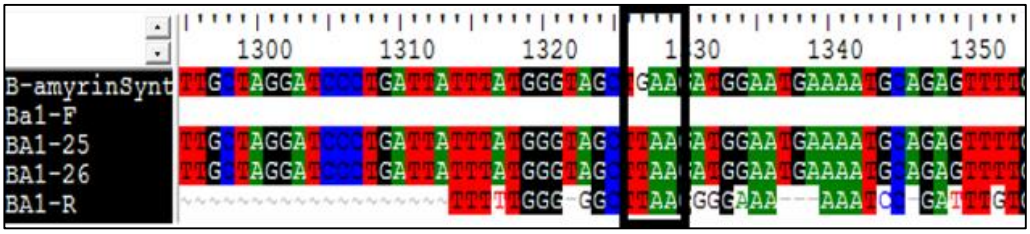


**Figure 15. β-amyrin synthase (βAS) sequence data**

Premature STOP mutation highlighted = GAA -> TAA.

Gateway Technology (LR reaction) was used to clone from the entry vector (pCR8/GW) into the destination vector (pEAQ-*HT*-DEST2)). The pEAQ-*HT*-DEST2 plasmid carrying the βAS gene was

then transformed into *Agrobacterium tumefasciens* for infiltration into *N. benthamiana* for transient expression.

### 3.2.4.2 Cloning of Cycloartenol Synthase

A putative cycloartenol synthase (CAS) was also selected as a candidate for cloning due to the structural similarities observed between cycloartenol and Solanoeclepin A. PCR of CAS was attempted using various PCR systems and primers described in the Materials and Methods section. However, PCR of this gene from potato cDNA template failed on multiple occasions, and therefore the gene was synthesised by IDTDNA using the transcript data available in the SpudDB database. As discussed, comparison of the SpudDB database with the NCBI database suggests incomplete annotation of this transcript sequence in the SpudDB database. For this reason, an N-terminal truncated version of CAS (1.5 kb) was originally cloned into pEAQ-*HT*-DEST2 (Figure 16).

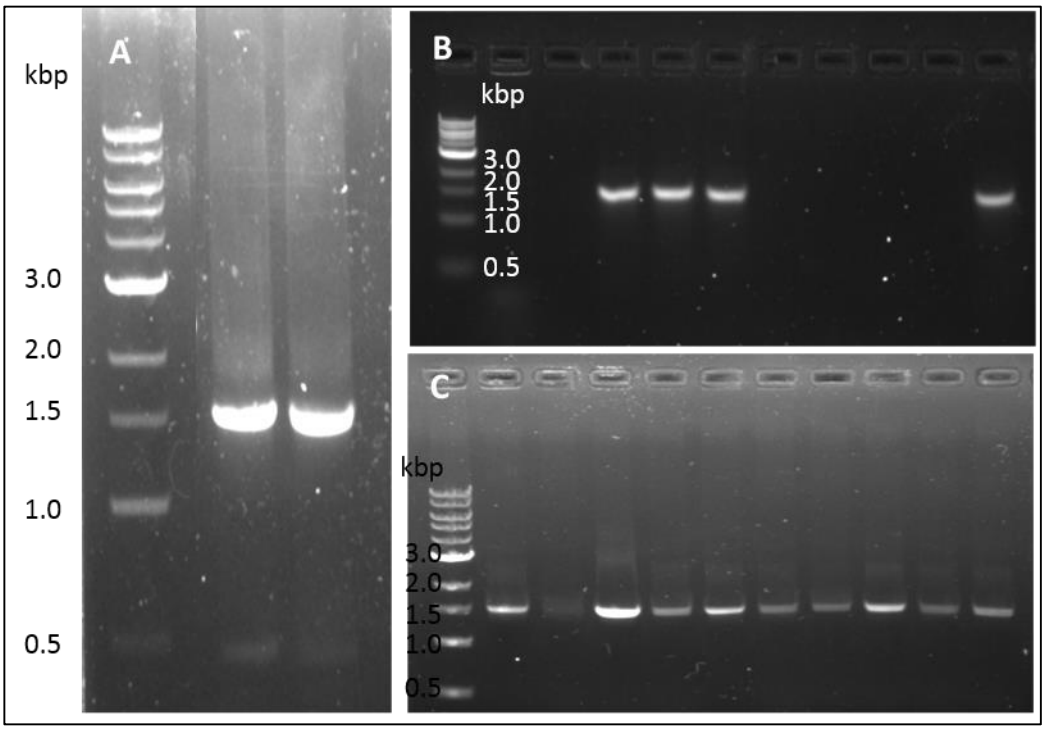


**Figure 16. Cloning of Cycloartenol synthase (CAS).**

Agarose gels displaying different cloning stages of N-terminal truncated cycloartenol synthase (CAS). (A) Cycloartenol synthase PCR product (1.5 Kb); (B) Cycloartenol synthase colony PCR following insertion into pCR8/GW vector; (C) Cycloartenol synthase colony pCR following ligation using LR reaction into pEAQ-*HT*-DEST2 vector.

Following discussion with the Osbourn group, it was determined that this gene sequence would produce a truncated version of the protein. Therefore the complete CAS gene sequence was identified from the NCBI database following detailed analysis of candidates. Gibson Assembly

(NEB) was used in order to produce the full length sequence from two synthesised fragments, and PCR amplification was used to increase concentration. Both TOPO cloning (into the pCR8/GW vector) and Gateway cloning (BP reaction into pDONR207 vector) were used for cloning. Cloning by both methods resulted in colonies growing on the respective antibiotic selection plates, however, sequence data in all cases could not be analysed due to poor read quality. Negative controls were carried out for cloning and transformation reactions, in which empty vector was transformed into *E. coli*, and these plates displayed no colonies, suggesting that the gene was inserted into the vector, however was not sequenced effectively (Figure 17).

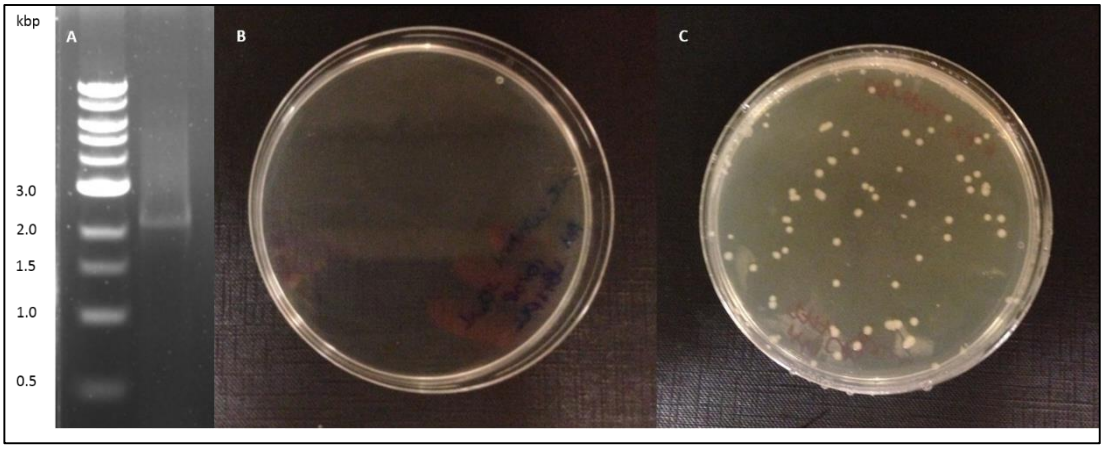


**Figure 17. Cloning of full length CAS gene.**

A. PCR product; B. Negative control spectinomycin selection plate; C. *E. coli* colonies transformed with CAS grown on spectinomycin selection plate.

Whilst CAS was not successfully cloned into pEAQ-*HT*-DEST2, the correct size PCR band was amplified, and sequencing data suggested that this PCR product was correct (Figure 17A).

### 3.2.5 Heterologous Expression and Analysis of OSCs in *N. benthamiana*

The recombinant plasmids with the desired genes were heterologously expressed in *N. benthamiana* in order to determine their impact on the metabolite profile of *N. benthamiana* by Gas Chromatography – Mass Spectrometry (GC-MS). Therefore, the background triterpene metabolite profile of *N. benthamiana* was determined by GC-MS, along with the triterpene metabolite profile of a GFP-expressing infiltrated control (Figure 18).

Table 30 identifies the differences between the uninfiltrated leaf and the GFP-infiltrated leaf triterpene metabolite profile. Differences in profile are highlighted in green. Identification of the compounds in this table was as a result of analysing mass spectra through the NIST 2.0 library search function.

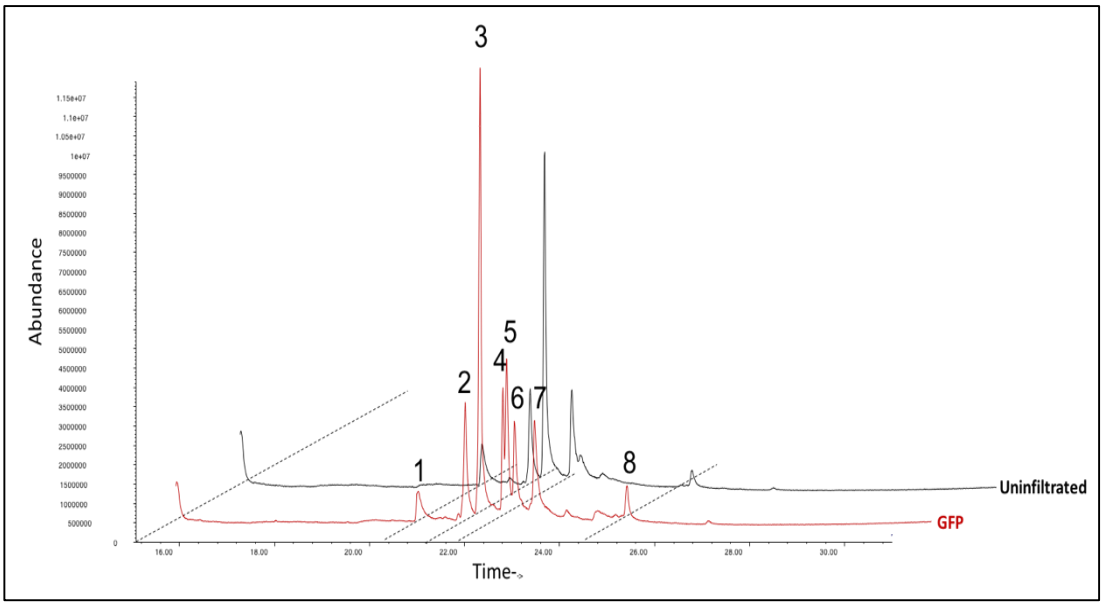


**Figure 18. GC-MS Triterpene Chromatogram for *N. benthamiana.***

GC-MS Total Ion chromatograms comparing background triterpene profiles between GFP-expressing and uninfiltrated leaves. Peak numbers correspond to numbers in Table 30.

**Table 30. Triterpene Metabolite Profile**

Differences between triterpene metabolite profile of uninfiltrated leaf and GFP-infiltrated leaf. Differences in metabolite profile are highlighted in green.
Y = present in chromatogram, N = not present in chromatogram.

| Chromatogram Number | Metabolite | GFP | Uninfiltrated |
|---|---|---|---|
| 1 | Cholesterol (derivatised) | Y | Y |
| 2 | Campesterol (derivatised) | Y | Y |
| 3 | Stigmasterol (derivatised) | Y | Y |
| 4 | Silane | Y | N |
| 5 | B-sitosterol (derivatised) | Y | Y |
| 6 | Stigmasterol | Y | Y |
| 7 | Cyclolanosterol | Y | N |
| 8 | Oleanolic acid | Y | Y |

### 3.2.6 Putative β-amyrin Synthase Expression Confirms Functional β-amyrin Synthase Activity

Heterologous expression of the putative βAS in *N. benthamiana* resulted in a significant peak in the chromatogram when analysed by GC-MS. This peak was not otherwise seen either in the uninfiltrated control, or in the control expressing GFP.

The β-amyrin chemical standard was observed in the chromatogram with a peak at 22.097 minutes and this clearly corresponded to the peak for the βAS-expressing leaf (Figure 19), suggesting that expression of βAS in *N. benthamiana* resulted in the production of β-amyrin. The nature of this peak was further confirmed as β-amyrin by the NIST 2.0 library search based on the Mass Spectrometry data acquired in GCMS analysis (Figure 20).

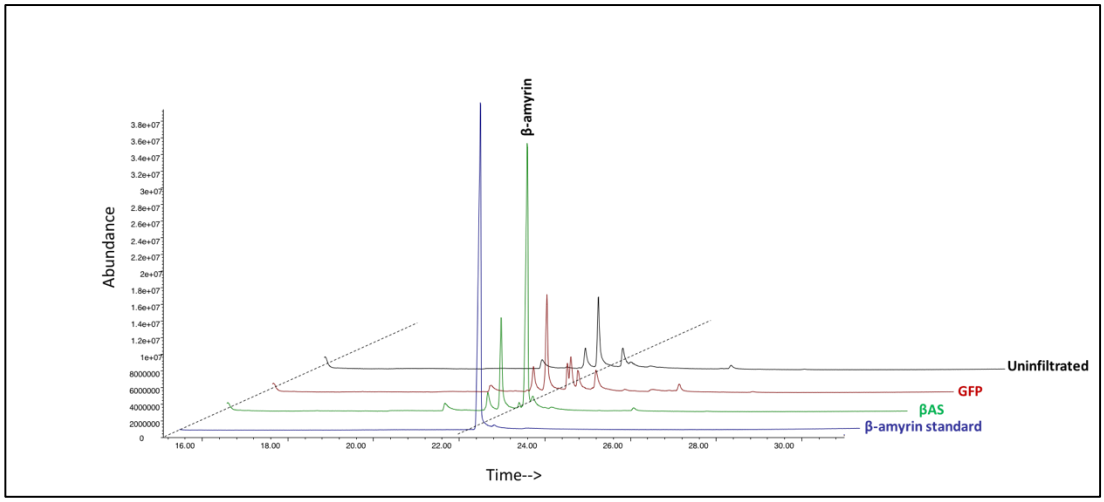


**Figure 19. GC-MS Triterpene chromatogram for infiltrated *N. benthamiana* leaves.**

GC-MS Total Ion Chromatogram comparing triterpene profiles across uninfiltrated, GFP-expressing, β-amyrin synthase expressing leaves and β-amyrin standard.

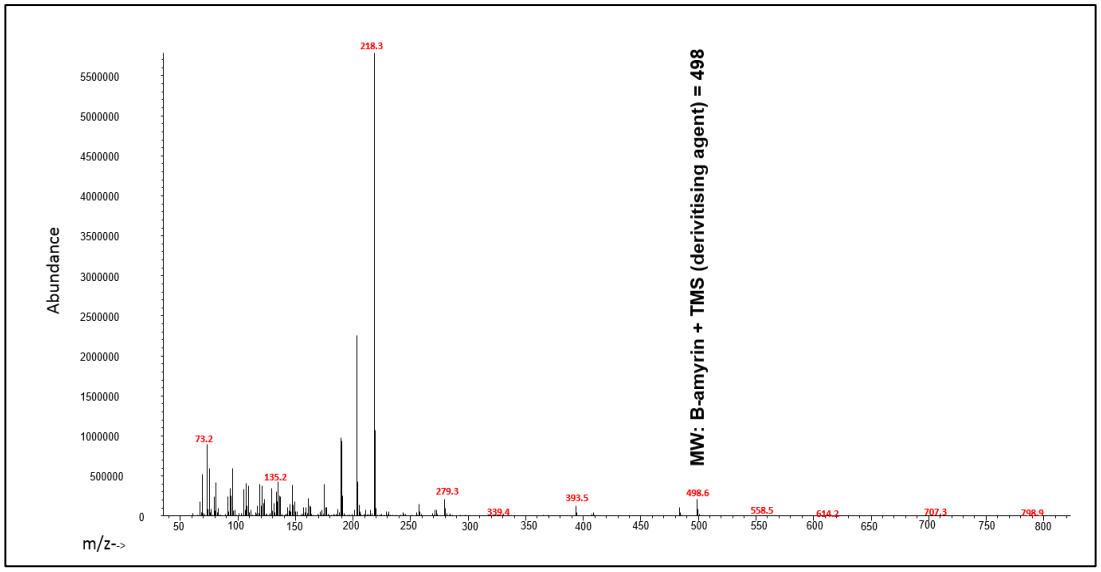


**Figure 20. Electron ionisation (EI) mass spectrum of B-amyrin.**

B-amyrin m/z = 426 (+ 72, as derivatised by TMS).

### 3.2.7 Characterisation of Cycloartenol GC-MS Profile

Although the predicted CAS gene is yet to be cloned into the pEAQ-*HT*-DEST2 vector which may then be used for infiltration of *N. benthamiana* leaves, the GC-MS profile of a cycloartenol

standard was characterised, which will provide a standard for comparison against when *N. benthamiana* leaves are infiltrated with CAS.

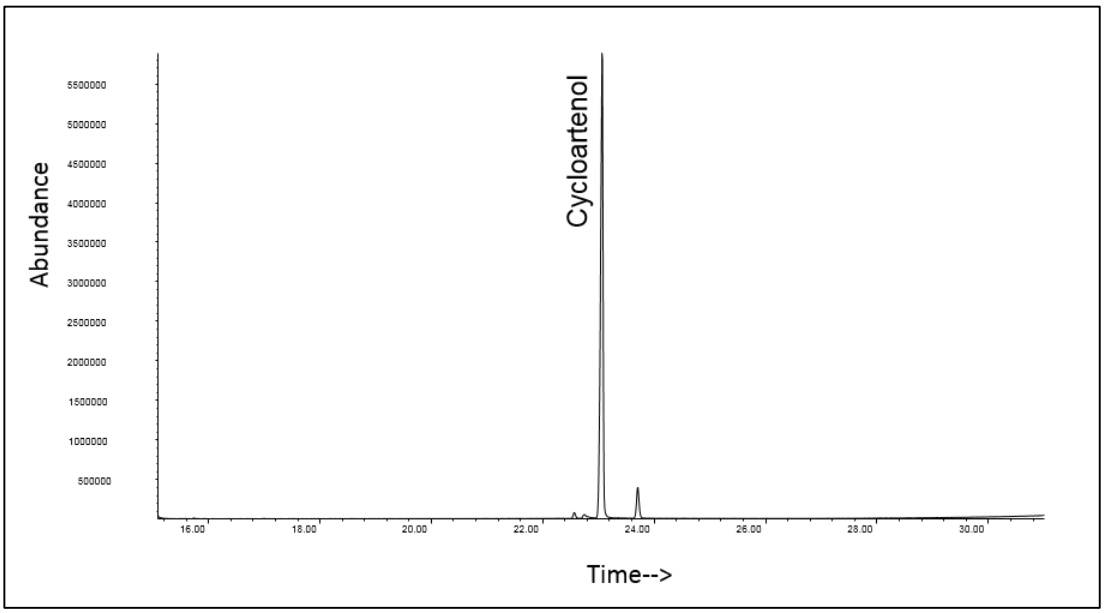


**Figure 21. GC-MS chromatogram of cycloartenol commercial standard.**

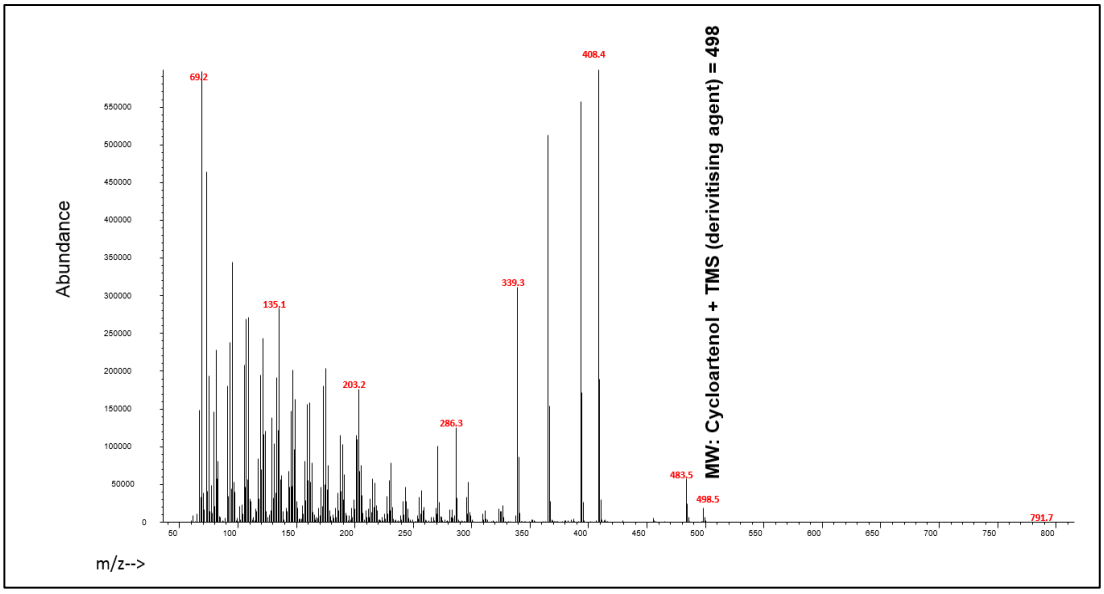


**Figure 22. Electron ionisation (EI) mass spectrum of cycloartenol.**

Cycloarternol m/z = 426 (+ 72, as derivatised by TMS).

A peak was observed at 23.12 minutes (Figure 21) and therefore it would be expected that a CAS-overexpressing leaf would display a corresponding peak at this time point. Identification of cycloartenol was confirmed by mass spectrometry, with an m/z of 498 (Figure 22).

## 3.3 Discussion

The potato genome is publicly available on the online database resource 'SpudDB'. This is a tool essential for carrying out studies of this sort. However it appears as though there may be inaccuracies in the gene annotation, therefore supporting the need for experimental confirmation. Identification of potato CAS homologs from the SpudDB database was problematic as the database identifies a truncated gene sequence as the full length CAS homolog. Upon more detailed analysis, and following communication with colleagues experienced in working with OSC genes, the possibility of the misannotion of a truncated gene was suggested. Analysis of the NCBI database for predicted potato CAS homologs led to the identification of the full length gene, which was then used in cloning. This example highlights the importance of detailed analysis of publicly available data resources, and suggests that other genes within the SpudDB genome database may also be inaccurately annotated.

Phylogenetic analysis of OSCs across an array of plant species suggests sequence conservation between different species. This is displayed in the Maximum Likelihood Tree (Figure 12) in which different OSCs cluster together by product type rather than by species. Predicted potato (*Solanum tuberosum*) β-amyrin synthase (βAS) and cycloartenol synthase (CAS) homologs follow this pattern and cluster with their respective groups, suggesting that potato OSCs are also closely related to the homologs of these genes present in other species. This suggests that the function of these putative potato OSCs is the same as the function observed in other species, however this requires experimental verification. As significant clustering of the gene homologs appears to occur across species, this may suggest that the evolution of these OSCs did not occur in the recent evolutionary past, and evolved in a common ancestor.

Differential expression of OSC genes was observed in potato through expression profiling of candidate genes. The fact that OSCs could not be amplified from all plant tissues suggests that not all potato tissues express OSCs. In some cases, such as that of lupeol synthase, the gene could only be amplified from the genomic DNA. This suggests that lupeol synthase is not expressed in root, shoot or leaf tissue under the conditions used in these experiments. This may not be surprising as OSCs are known to be involved in plant secondary metabolism (Haralampidis, Bryan et al. 2001); often associated with defence mechanisms. The expression of such genes may only be induced by stress, such as tissue damage or herbivory.

RNA from all tissue samples was extracted from the plant at the same time (3 weeks after initial culturing), which does not capture differences in expression over time. It is possible that there

is a temporal factor in the differential expression. To test this hypothesis, it may be interesting to extract RNA from the different tissue samples at regular intervals, for example at weekly intervals. Gene expression could then be compared both between both tissue samples and over a period of time. Potato plants could also be grown under environmental stresses. Expression of OSCs may then be characterised when the plant is under attack from pathogens, or when being grown under abiotic stress.

Cloning of candidate OSC genes in potato required significant optimisation, however this has allowed for the development of protocols for future work. Once the original CAS gene being worked with was found to be truncated, cloning of the full length gene was attempted. Synthesised gene fragments were assembled to produce the full length gene. This assembly was used in a PCR reaction and a single band of approximately 2.3 kb was observed, which would suggest that the assembly was successful. Both pCR8/GW and pDONR207 vectors were used in order to attempt to successfully clone this gene into *E. coli*. When competent cells were transformed with the vector carrying the CAS gene, *E. coli* colonies grew on antibiotic selection plates, which would suggest that the cells had been transformed with the vector. In the case of pCR8/GW, a gene insert is required for the vector to become circularised, and therefore expressed due to the mechanism of action of topoisomerases involved in this method of cloning. In this example, the gene may be inserted into the vector in the wrong orientation, and this highlights the importance of screening for colonies with the correctly oriented gene using colony PCR. As pDONR207 relies on homologous recombination through the BP reaction for ligation of the gene of interest into the vector, it is highly unlikely that the gene will insert in the wrong orientation. It is also unlikely the empty vector will re-ligate without a gene being inserted. In order to test whether this may be the case, and the reason for receiving poor sequence data, a restriction digest could be used in order to determine whether the gene has been inserted or not.

To overcome the problems associated with sequencing these vectors, it may be necessary to use another alternative vector for cloning, or to try different sequencing methods, such as using a different company for sequencing, or by sending the plasmid directly for sequencing rather than carrying out the sequencing reaction in-house.

Heterologous expression of OSCs in *Nicotiana benthamiana* leaves allowed the characterisation of triterpene metabolite profiles. Crucially, it was essential to first characterise differences in triterpene metabolite profile between uninfiltrated *N. benthamiana* leaves and GFP-infiltrated

leaves. GFP was used as a control for this experiment as opposed to an empty vector as this meant that heterologous protein expression was occurring in the negative control leaves. Therefore, any alterations in triterpene metabolism associated with the effect of heterologous protein expression was accounted for with this control. Following analysis by GC-MS, and using the NIST 2.0 library search function, it appears as though uninfiltrated leaves and GFP-infiltrated leaves show subtle differences in their triterpene metabolite profile. These differences should be accounted for when analysing triterpene metabolite profiles of OSC-infiltrated leaves.

When comparing the βAS-expressing leaf chromatogram to the chromatogram of the GFP-expressing leaf, the difference occurs at a retention time of 22.1 minutes and is due to the presence of a peak representing β-amyrin. This suggests that overexpression of βAS in *N. benthamiana* results only in the production of β-amyrin, as opposed to other metabolites, which would have been displayed on the chromatogram as other peaks. Therefore it can be concluded that the function of this putative βAS homolog in potato is to synthesise β-amyrin. It may also be concluded that βAS is unlikely to be involved in Solanoeclepin A biosynthesis as an enzyme synthesising alternative products, which may be involved in Solanoeclepin A biosynthesis. However, this does not rule βAS out of Solanoeclepin A biosynthesis entirely as β-amyrin may be involved as a direct precursor of Solanoeclepin A. In this case, β-amyrin would be modified by downstream enzymes in the Solanoeclepin A biosynthesis pathway. In order to test this hypothesis, candidate downstream enzymes could be co-expressed in *N. benthamiana* and GC-MS analysis carried out to determine whether β-amyrin is used as a substrate for modification by candidate enzymes. It should also be noted that not all compounds will fly as a result of ionisation when analysed by GC-MS, and therefore further analysis may be necessary. The use of a shorter column (10m), as opposed to the 30m column described in Materials & Methods may be useful to ensure that all compounds have been removed from the column.

As CAS is yet to be overexpressed in *N. benthamiana*, it is unclear whether CAS is likely to play a role in Solanoeclepin A biosynthesis. Once CAS has been overexpressed in *N. benthamiana* it will be interesting to compare the triterpene metabolite profile of CAS-infiltrated leaves along with uninfiltrated and GFP-infiltrated leaves and against the cycloartenol chemical standard in order to determine whether peaks other than the one observed for cycloartenol are present. If this is the case, metabolites other than cycloartenol may be observed, suggesting that CAS displays promiscuity by producing alternative products. If cycloartenol is a direct precursor for Solanoeclepin A, it may be necessary to co-express candidate downstream modification genes along with CAS.

It should be noted that cycloartenol is produced by *N. benthamiana* and that quantitation of peaks in the chromatogram corresponding to cycloartenol will be necessary to determine whether CAS has been overexpressed in infiltrated leaves. Alternative expression systems, which do not produce cycloartenol, such as yeast could also be used. Whilst identifying whether secondary peaks are present following overexpression of CAS in *N. benthamiana,* it should also be noted that CAS is a highly conserved enzyme amongst plant species. Therefore, if it is involved in Solanoeclepin A biosynthesis in the potato, it is likely to be a very early step in this pathway. This may be of interest when looking for potential methods for alleviating the problems caused by PCN, as manipulation of an enzyme that plays a role early in the biosynthesis of the compound of interest is less likely to result in the accumulation of potentially toxic intermediates downstream.

This study hypothesised that OSCs are involved in the biosynthesis of Solanoeclepin A in potato, due to their involvement in triterpene biosynthesis. A putative potato βAS has been cloned and heterologously expressed in *N. benthamiana*. Analysis by GC-MS suggests that expression of this gene results in β-amyrin production in the leaves. This indicates that this predicted βAS gene has the same function as its homologs in other species, but may not be directly involved in the biosynthesis of downstream metabolites, such as Solanoeclepin A.

CAS was hypothesised as a possible candidate for Solanoeclepin A biosynthesis, and cloning and expression of this gene has been attempted. Further work is required to optimise this cloning method before it may be expressed in *N. benthamiana* to determine its role in potato triterpene biosynthesis. With regards to Solanoeclepin A biosynthesis, triterpene cyclases other than those studied in this work should also not be ruled out without further investigation and experimentation.

### 3.3.1 **Future Work**

As the predicted potato CAS gene is yet to be cloned and expressed in *N. benthamiana*, this should be carried out as a matter of priority in order to determine the function of CAS in potato, and its likely involvement in Solanoeclepin A biosynthesis. Other candidate OSCs should also be heterologously expressed in *N. benthamiana* to determine the function of these putative potato OSC homologs. Following this, elucidation of downstream steps in the biosynthesis pathway will be useful in order to gain an insight into how the potato plant synthesises Solanoeclepin A.

Elucidating the biosynthesis pathway may also indicate the reason for the plant producing the compound, and therefore suggest how the plant may be affected if Solanoeclepin A biosynthesis were to be inhibited.

# 4 Chemical Inhibition of Potato Sprouting in Storage Conditions

## 4.1 Introduction

Potato tubers undergo a period of dormancy once harvested, following which they begin to sprout. This section will look at the process of tuber sprouting, along with chemical inhibitors which may be used in the potato farming industry to prevent sprouting.

### 4.1.1 Tuber Development

Potato tubers are formed as a result of the development of underground shoots, known as stolons, and tuberisation at the stolon tip (Booth 1963). Stolons develop from lateral underground buds due to transverse cell divisions and cell elongation in the apical region of buds, however tuber formation begins when stolon elongation stops and the pith and cortex cells become enlarged and begin to divide longitudinally. This results in stolon tip swelling (Xu, Vreugdenhil et al. 1998). Following tuber initiation, the bulk of the tuber tissue is then formed as a result of cell expansion and randomly oriented cell division (Jackson 1999) as well as a large deposition of starch and storage proteins (Visser, Vreugdenhil et al. 1994). Tuber development is regulated by a complex interaction of endogenous and environmental signals and the process is ultimately controlled by coordinated transcriptional and metabolic changes (Kloosterman, Vorst et al. 2005, Kloosterman, De Koeyer et al. 2008). Environmental signals are key for tuber development induction, and under appropriate conditions, a tuberisation signal is produced in the leaves. This is then transported to the stolons in order to induce tuber formation. Interestingly, the nature of this tuberisation signal has been identified as *StSP6A*, and is the potato homolog of the *Arabidopsis thaliana Flowering Locus T* (*FT*) (Navarro, Abelenda et al. 2011).

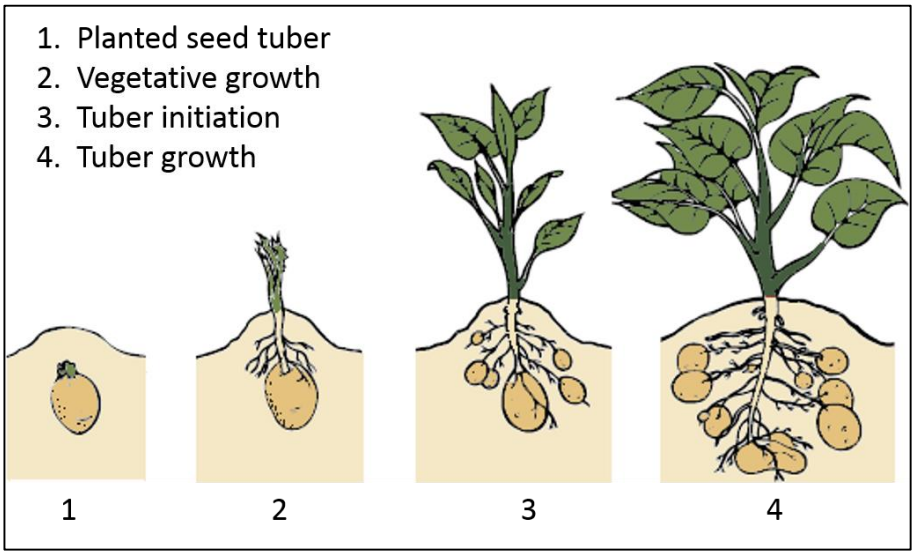


**Figure 23. Stages of potato development.**

Pictures taken from Food and Agriculture Organisation of the United Nations – International Year of the Potato. (Nations 2009)

Upon reaching physiological maturity, potato tubers enter a period of dormancy, during which tubers will not sprout even if placed in ideal conditions for germination. Meristematic activity in the stolon apex and nodes ceases, meaning that the tuber enters this period of dormancy (Burton 1989). Therefore, the start of dormancy is coincident with tuber enlargement (Fernie and Willmitzer 2001).

### 4.1.2 Dormancy

Dormancy is defined as 'the absence of visible growth of any plant structure containing a meristem' (Lang, Early et al. 1987). Dormancy in potato tubers occurs in the tuber buds containing the meristem, whereas the rest of the tuber remains metabolically active (Viola, Pelloux et al. 2007). Dormancy in potato tubers is split into three phases (Suttle 2007): endodormancy, para-dormancy and ecodormancy. Endo-dormancy occurs after harvest and arises from factors within the affected organ as opposed to external causes (Suttle 1998). Eco-dormancy is the effect of environmental conditions such as low temperature causing a delay in sprouting (Mani, Bettaieb et al. 2014). Para-dormancy is signalled from an area of the plant different to where dormancy occurs, an example of this being apical dominance: the situation in which the apical meristem impedes the development of sprouts or a secondary bud (Mani, Bettaieb et al. 2014). Following the endo-dormancy period, tubers become physiologically capable of sprouting. Therefore the length of dormancy period following endo-dormancy varies between tubers of the same variety. Indeed, due to the poor understanding of mechanisms controlling potato tuber dormancy, it is still unclear as to whether the growth of buds is

regulated within the bud itself (endodormancy), or if this regulation comes from the rest of the tuber (paradormancy) (Viola, Pelloux et al. 2007).

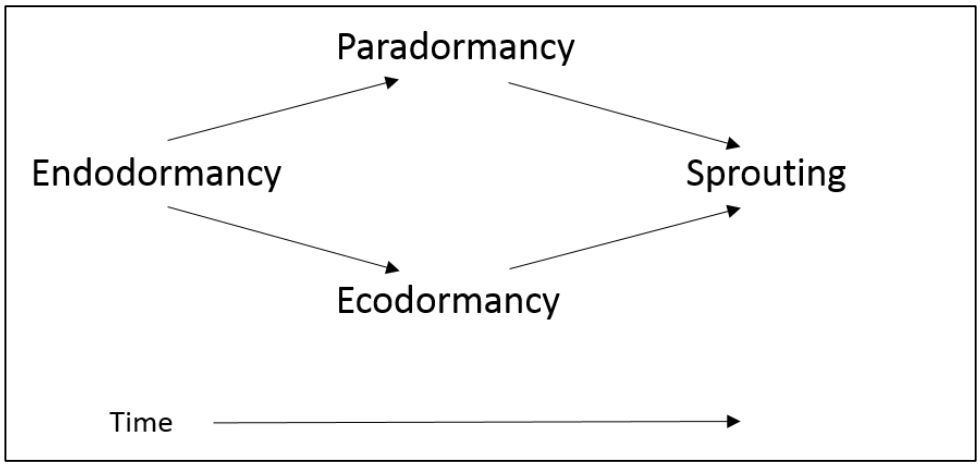


**Figure 24. Stages of tuber dormancy.**

The period of dormancy confers a biological advantage to potato plants, and this relates to the survival of the species. Dormancy allows potato varieties to overwinter, meaning that they only resprout and therefore reproduce again in the spring, once the unfavourable winter conditions have passed (Suttle 2007, Mani, Bettaieb et al. 2014). Varieties with a dormancy period are more likely to be reproductively successful, and therefore be selected for, as the chance of survival is greater when offspring have favourable conditions in which to grow. Furthermore, the dormancy period of the tuber is exploited by farmers, who may need to store potatoes for several months before tubers go to market. This prolongs the period in which potatoes can be stored without sprouting control compounds, and therefore farmers may choose to grow varieties with longer endo-dormancy periods or with eco-dormancy periods that are easier to influence, as this results in lower costs for storage.

### 4.1.3 Regulation of Dormancy Period and Tuber Sprouting

Sonnewald and Sonnewald reviewed the regulation of potato tuber sprouting in 2014 and highlighted the key factors influencing dormancy break and sprouting onset, and these include environmental, metabolic, hormonal, structural, cellular and transcriptional factors. Comparative transcript analysis revealed the differential expression of a significant number of genes when comparing growing and dormant tubers, however a 'master regulator' of sprouting is yet to be identified (Sonnewald and Sonnewald 2014).

#### 4.1.3.1 Environmental Factors Affecting Tuber Dormancy and Sprouting

Various environmental factors affect dormancy period length, including both during the tuberisation period when dormancy is established, and during post-harvest storage. The

negative effects of high temperature, short day length, and cycling between high and low nutrient levels on dormancy and sprouting have been discussed (Jackson 1999, Claassens and Vreugdenhil 2000, Suttle 2007). Once potato tubers are in storage, temperature is a critical factor in determining dormancy period length. Humidity and atmospheric composition are also important for dormancy (Suttle 2007), and therefore farmers maintain tight regulation over their storage units in order to minimise losses.

#### 4.1.3.2 Primary Metabolism

Significant changes also occur in the plant's primary metabolism during tuber development and at the onset of sprouting. Once the tuber has been detached from the mother plant, the tuber undergoes a rapid transition from acting as a sink organ, in which starch synthesis occurs, to becoming a source organ mobilising starch reserves to fuel sprout development (Viola, Pelloux et al. 2007) (Figure 25).

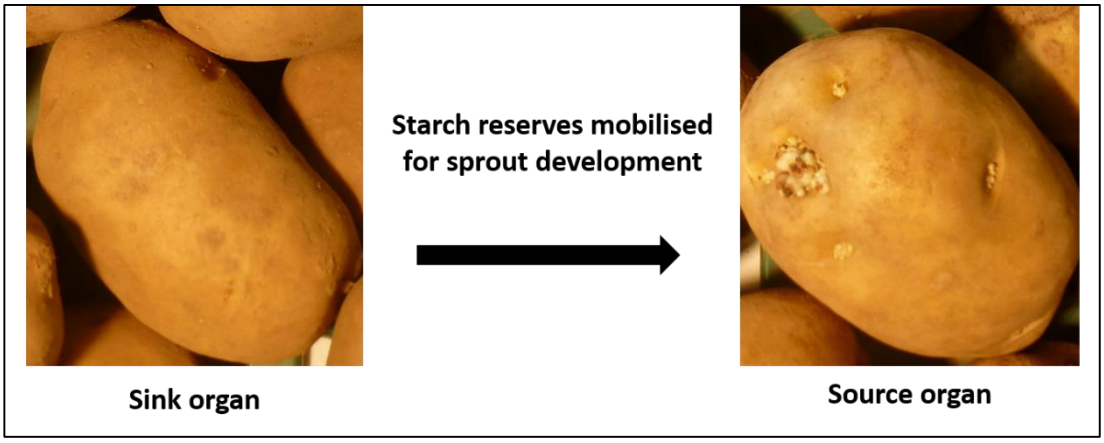


**Figure 25. Potato sprout development.**

Transition of potato tuber from sink organ to source organ at the break of dormancy.

Such drastic changes as the sink-source transition occur rapidly over a period of a few days. Viola *et al.* also demonstrated that metabolite pools in growing buds experience significant increases in starch and sucrose levels (Viola, Pelloux et al. 2007). Furthermore, the activity and transcript abundance of α- and β- amylases were observed to increase in sub-eye regions of the tuber upon the onset of sprouting (Biemelt, Hajirezaei et al. 2000, Rentzsch, Podzimska et al. 2012). This suggests that there is an increase in the turnover of starch in meristematic tissue, and the degradation of starch is important in order to maintain sprout growth (Sonnewald and Sonnewald 2014). Extensive transgenic studies have been carried out in order to manipulate the mobilisation and metabolism of sucrose for the purpose of exploiting the onset of sprouting. As has been discussed, the tuber shifts from acting as a sink organ to being a source organ at the break of dormancy and onset of sprouting. The tuber displays an altered structure dependent

on its developmental stage. Viola *et al.* (2007) demonstrated that the apical bud remains symplastically isolated in developing tubers and in tubers immediately after harvest, however in growing buds the symplastic connection is re-established. 'Symplastic gating' modulates the supply of metabolites to the meristem, and this affects meristem activity(Viola, Pelloux et al. 2007). Therefore, structural changes in the tuber, such as those described here, play a role in controlling the onset of sprouting, as they control the transport of metabolites such as sucrose into the meristem. This shift towards the mobilisation of sucrose from starch has already been outlined as being important in the control of dormancy break.

As the mobilisation and availability of sucrose is essential for bud dormancy break, sucrose could be identified as a signalling molecule for the process of sprouting onset. Trehalose-6-phosphate (T6P) has been identified as part of the plant's sugar signalling system (Paul, Primavesi et al. 2008, Debast, Nunes-Nesi et al. 2011), however studies into the role of T6P have produced inconsistent results, and therefore the role of T6P in sugar signalling for dormancy break remains to be elucidated. Interestingly, however, Debast *et al.* (2011) showed that tubers with low levels of T6P sprouted early, whilst those with high T6P levels were delayed in sprouting. Tubers with modified T6P levels had altered gibberellin and cytokinin responses and altered turnover of abscisic acid. Abscisic acid interacts with SnRK1 (SNF1-related kinase 1) – a crucial player in sugar signalling – and therefore it has been hypothesised that the induction of ABA catabolism due to SnRK1 signalling in plants with modified T6P levels may cause premature sprouting (Debast, Nunes-Nesi et al. 2011). This example demonstrates the complex interplay between different factors; here primary metabolism and hormonal controls interact, in order to regulate bud dormancy break and sprouting onset.

#### 4.1.3.3 Hormonal Control

Hormonal regulation is also crucial in regulating tuber development, dormancy initiation and the onset of sprout development. However the literature often appears to contradict itself, with much debate around the specific roles of phytohormones in tuber dormancy and sprout development. Several models attempting to outline hormonal regulation of tuber dormancy and sprouting have been published, however it is clear that this work is far from complete. Understanding and characterising the roles played by phytohormones may be challenging due to the complex interactions that occur between hormones. In addition to this is the fact that the studies carried out to date have used tissue samples from different parts of the plant as well as of differing developmental stages, and in many cases have used different strategies for elucidating the role of phytohormones. The five major plant hormones: Abscisic acid, ethylene,

gibberellins, cytokinins and auxin, have all been implicated in playing a role in tuber dormancy and sprouting, however the extent to which each is involved differs significantly.

Abscisic acid has been shown to play a crucial role in both initiation and maintenance of tuber dormancy. Characterising the role of ABA was carried out using a variety of techniques, including inhibitory studies (Suttle and Hultstrand 1994), exogenous ABA application (Suttle, Abrams et al. 2012), qRT-PCR of ABA biosynthesis genes (Destefano-Beltrán, Knauber et al. 2006), and transgenic studies (Debast, Nunes-Nesi et al. 2011, Suttle, Abrams et al. 2012).

Literature regarding the role of ethylene in dormancy initiation appears to be conflicting. However, Suttle (2007) reviewed these findings, and in summary it appears as though ethylene plays a crucial role in tuber dormancy induction (Suttle 2007). Ethylene production appeared to be highest during the initial two weeks of *in* vitro culture, after which its production decreased (Suttle 1998). Inhibition of ethylene through the use of silver nitrate resulted in a dose-dependent increase in sprouting during the initial period of *in vitro* culture, as opposed to throughout their culturing, therefore suggesting that endogenous ethylene plays a role in the induction of tuber dormancy (Suttle 2007). Hartmann *et al.* (2011) demonstrated that ethylene signalling may negatively influence sprout growth through the comparison of sprouting tubers with non-sprouting tubers (Hartmann, Senning et al. 2011). This further supports the hypothesis that ethylene is involved in tuber dormancy.

In contrast to the proposed roles of ABA and ethylene in tuber dormancy induction and maintenance, gibberellins (GA) and cytokinins (CK) are hypothesised to have roles associated with the break of dormancy and onset of sprouting. GA is well documented in having the ability to force the break of dormancy in tubers and to stimulate the onset of sprouting (Brian, Hemming et al. 1955), and this has been confirmed more recently through treatment with bioactive GA species (Suttle 2004, Hartmann, Senning et al. 2011). Transgenic studies carried out by Hartmann *et al.* (2011), whereby an *Arabidopsis GA2-oxidase* was expressed in tubers, support the role of GA as involved in dormancy termination (Hartmann, Senning et al. 2011). Interestingly, endogenous contents of specific GAs was found to be highest during the period of sprout growth and lowest during storage when tubers are dormant (Suttle 2004). The fact that GA level is highest during robust sprout growth and not immediately at the break of dormancy may also implicate GA in sprout growth.

Cytokinins (CK) are believed to play an essential role in the control of dormancy and sprouting, and this hypothesis is supported by findings from Hartmann *et al*. (2011), who showed that expressing cytokinin oxidase/dehydrogenase 1 (CKX1) from *A. thaliana* resulted in tubers which displayed a prolonged dormancy and a delay of up to 8 weeks for sprouting onset compared to the wild type (Hartmann, Senning et al. 2011). In addition, it has been shown that prolonging the storage period led to an increase in cytokinin sensitivity that correlated with increase in time spent in storage (Suttle 2001), hence suggesting that cytokinins are involved in dormancy break. These findings, along with the fact that cytokinins are defined by their ability to stimulate cell division, support Suttle's (2007) statement declaring cytokinins as 'cognate regulators of tuber dormancy exit in potatoes' (Suttle 2007).

Despite the key role of auxin as a plant regulator, its role in tuber dormancy and sprouting onset is poorly understood. Studies carried out by Sorce *et al.* found a positive correlation between the content of IAA in tuber buds and the loss of dormancy (Sorce, Lorenzi et al. 2000), and a decline in the levels of free and conjugated IAA in tuber buds until the commencement of tuber sprouting (Sorce, Lombardi et al. 2009). Further studies summarised by Sonnewald and Sonnewald (2014) suggest that the biosynthesis of auxin, along with its transport and signalling, may be required for cellular differentiation during bud break and sprout outgrowth, however further studies need to be carried out to confirm these findings (Sonnewald and Sonnewald 2014).

Clearly, extensive studies have been carried out to determine the role of hormonal control during the processes of tuber dormancy and sprouting, and these suggest that ABA and ethylene are involved in the induction of dormancy, whilst gibberellins, cytokinins and auxins play a role in dormancy break and sprouting growth. Whilst several models have been proposed that outline hormonal control, further investigations are required to clarify the specific roles of each hormone.

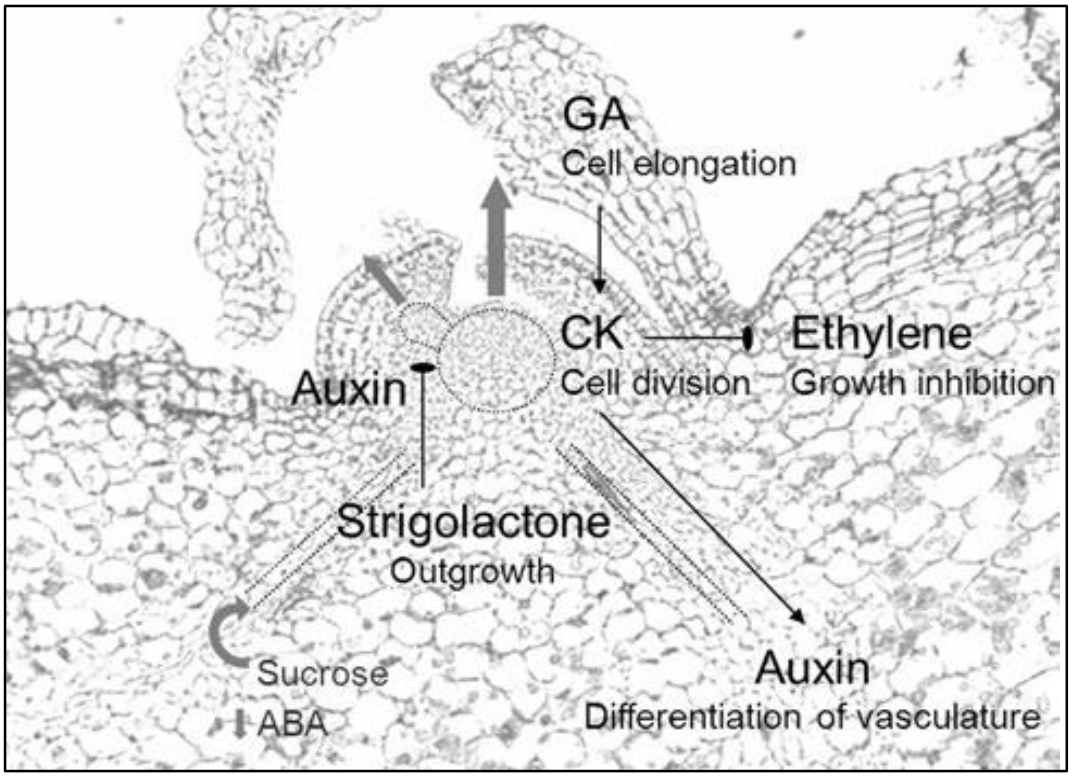


**Figure 26. Hormonal regulation of tuber dormancy model.**

Model of hormonal regulation of tuber dormancy and sprouting as proposed by Sonnewald & Sonnewald 2014. Role of plant hormones in bud tissue dormancy and sprout tissue development. Cytokinins are proposed to have a key role in stimulating cell division, whilst gibberellins and auxins also play a role in sprout growth. Ethylene and Abscisic acid are proposed to be involved in the induction and maintenance of tuber dormancy. Figure reproduced from Sonnewald & Sonnewald 2014 (Sonnewald and Sonnewald 2014), with permission from Springer.

#### 4.1.3.4 Cellular and Transcriptional Control

Cellular and transcriptional changes have also been shown to occur through the progression of tuber development, dormancy and sprout growth, and these changes play a significant role in the control of tuber dormancy break and sprouting onset. During the dormancy period, cells do not replicate and this is explained by the fact that nuclei from meristematic cells of dormant tubers have an arrested cell cycle in the $G_1/G_0$ stage (Campbell, Suttle et al. 1996). At the onset of sprout growth, meristematic activity is re-activated, and this is accompanied by an increase in cell division, due to the cell cycle no longer being arrested at this stage (Sonnewald and Sonnewald 2014).

Epigenetic changes have also been shown to be involved in the regulation of dormancy. Studies in which tubers were treated with bromoethane, in order to stimulate premature dormancy break, displayed a transient increase in histone acetylation (David Law and Suttle 2004), and a decrease in cytosine methylation has also been linked to an increased rate of cell division (Law

and Suttle 2003). Therefore, it may be concluded that an increase in cytosine methylation and histone deacetylation form a defined sequence of epigenetic changes, which ultimately result in tuber meristem re-activation, and therefore are involved in tuber dormancy break and sprout growth (David Law and Suttle 2004). A decrease in these mechanisms occurs before an increase in RNA and DNA synthesis (Sonnewald and Sonnewald 2014).

Various studies have been carried out to determine differential gene expression during the periods of dormancy and sprout growth. A study carried out by Liu *et al.* (2012) identified 304 expressed sequence tags (ESTs) associated with tuber dormancy release. qRT-PCR analysis of 14 transcripts identified 13 candidate genes as being significantly upregulated in the progression of the tuber through dormancy to sprouting. Tissue specific expression of one gene identified – ADP-ribosylation factor (*ARF1*) gene – suggested that its expression was highest in the tuber, and this increased significantly after tuber dormancy break, suggesting its likely involvement in tuber dormancy and sprouting (Liu, Zhang et al. 2012).

However, despite these cellular and transcriptomic studies, the understanding of key regulators of tuber dormancy and sprouting onset are still as yet unknown. Further studies into the environmental, structural, metabolic, hormonal and cellular controls over the processes of dormancy and sprouting should ultimately result in a clearer understanding of the molecular mechanisms underlying bud break and tuber sprout initiation (Sonnewald and Sonnewald 2014).

### 4.1.4 Significance of Dormancy and Sprouting Control on Potato Crop and Storage

An understanding of the key regulatory processes controlling tuber dormancy and sprouting onset is crucial as potatoes must be stored year round in order for growers to meet demand from consumers. Therefore, understanding how dormancy is regulated allows farmers and breeders to exploit these factors and prolong the period of dormancy before sprouting onset. Farmers can exploit these factors in several ways in order to minimise the losses to their crop due to sprouting such as: selecting varieties with a long endodormancy period, controlling the environment in which tubers are stored to prolong the ecodormancy period, and deciding when the optimal time is to send varieties that have been stored to market.

**Figure 27. Potato storage unit.**

Representative of stores used in the industry.

Low storage temperatures minimise the extent to which tubers sprout, and therefore growers tend to keep their storage facilities cold. However, if storage temperatures are too low, reducing sugars and acrylamide – a carcinogen - may accumulate in the tubers. This process is known as cold-induced sweetening. When these tubers are processed at high temperature, the resulting product is dark coloured and has a bitter taste, which is undesirable to processers and to the consumer. This is due to a change in the flux between starch and sucrose, during which the net rate of sucrose synthesis increases. These changes in end product quality need to be mitigated, and this means that growers must not allow their storage facilities to become too cold. Studies have been carried out to determine the genes controlling cold-induced sweetening, and this understanding may be exploited to develop varieties which are less susceptible to cold-induced sweetening. For example, transgenic tubers encoding a vacuolar homolog of a tobacco cell wall invertase inhibitor resulted in up to a 75% decrease in cold-induced hexose accumulation (Greiner, Rausch et al. 1999).

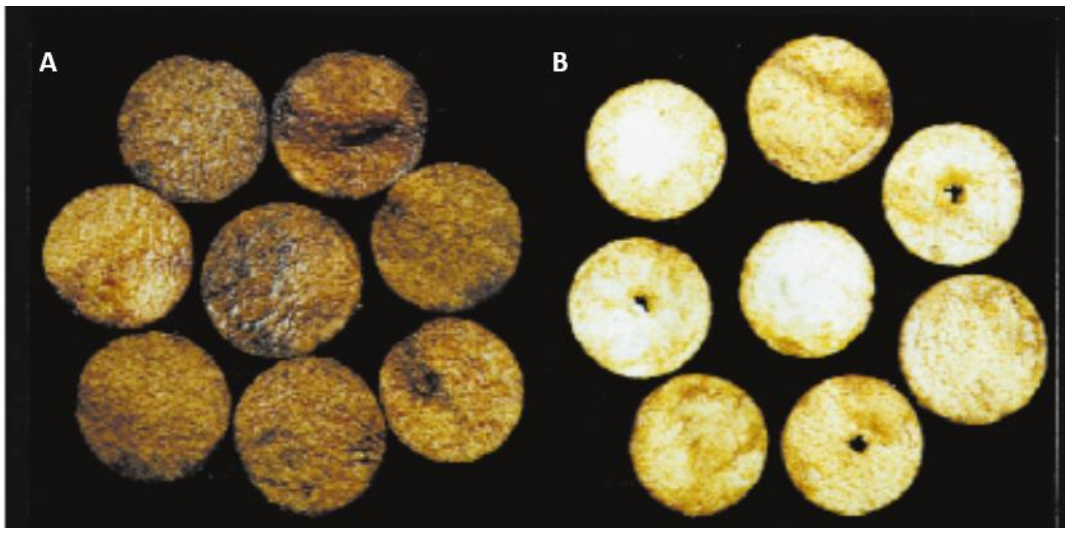


**Figure 28. Expression of tobacco cell wall invertase inhibitor.**

Effect of expression of tobacco cell wall invertase inhibitor on cold-induced hexose accumulation in potato. (A) Wild Type; (B) transgenic tuber. Wild type shows effect on processing quality when stored at 4 $^{o}$C. Figure and data taken from (Greiner, Rausch et al. 1999). Reproduced from Greiner *et al.* (1999), with permission from Nature Publishing Group.

Potato breeders have long been attempting to develop a 'cold-chipper': a cultivar suitable for processing (frying) that does not accumulate reducing sugars when stored in the cold. However whilst progress has been made and cultivars exist which accumulate low levels of reducing sugar (Hamernik, Hanneman et al. 2009), there are no varieties that may be stored at cold temperatures and not undergo cold-induce sweetening (Bhaskar, Wu et al. 2010). However, Hamernik *et al.* (2009) identified diploid wild *Solanum* species accessions that do appear to be resistant to cold-induced sweetening, even at temperatures as low as 2 °C. Accessions were crossed with haploids of *S. tuberosum* and several hybrids produced chips of an acceptable quality following 3 months of storage at 2 °C. The best wild species parents were shown to be *S. raphanifolium* accessions (Hamernik, Hanneman et al. 2009). Further to this, fine screening has been carried out to identify phenotypic variability for resistance to cold-induced sweetening between individuals of *Solanum raphanifolium*. It was revealed that resistance to cold-induced sweetening was dependent on the individual as different individuals showed different levels of resistance. This highlights the importance of fine-screening in order to select individuals in potato accessions that show the greatest resistance (Ali and Jansky 2015).

Bhaskar *et al.* (2010) demonstrated that silencing of the potato vacuolar acid invertase (*VInv*) gene prevents the accumulation of reducing sugars in cold-stored tubers and high-temperature processing of these lines also showed a 15-fold acrylamide reduction and were light in colour, despite being stored at 4 °C (Bhaskar, Wu et al. 2010). Therefore, the threat to processing

quality and build-up of harmful metabolites due to cold-induced sweetening may be overcome through the production of transgenic lines, or through targeted breeding programmes.

### 4.1.5 Storage of Tubers for the Crisping Industry

Approximately 500,000 tonnes of potatoes are grown for the crisping industry each year in the UK, making up around 10% of the total potato crop grown. However, despite this seemingly small proportion of the total crop, £2 billion is spent each year in the UK on crisps and potato snacks, accounting for approximately 50% of the total consumer spend on potato products (Personal Communication(Hewitt 2015)). Therefore, crisps are clearly a high value product, making up a significant part of the market for potato products and are highly valuable to the grower's economy (Personal Communication(Hewitt 2015)).

Prevention of sprouting is of importance for farmers growing potatoes for the crisping industry as dormancy break and sprouting onset is associated with the breakdown of starch to reducing sugars, as has been detailed. Such breakdown of starch leads to a darkened processed product and a bitter taste, which are both undesirable to the consumer. Therefore, if growers allow sprouting to occur to too great an extent, their crop is less valuable and may even be rejected by the processor. Prevention of sprouting is also necessary from the processor's perspective due to the limitations of the machinery which is used to process tubers, and from the consumer's perspective due to aesthetic reasons.

For these reasons, the inhibition of sprouting is highly important to potato growers in order to ensure their crop has maximum value.

### 4.1.6 Conventional Chemical Inhibitors of Sprouting

Along with exploiting the endodormancy and ecodormancy periods of potatoes, as has been discussed, farmers also use chemical inhibitors of sprouting to ensure that minimal sprouting occurs. Such chemical inhibitors should be economically viable, as they must be applied on a very large scale.

Chlorpropham (CIPC), isopropyl-3-chlorophenyl carbamate, has traditionally been used as a chemical inhibitor of potato sprouting to increase the storage period of tubers.

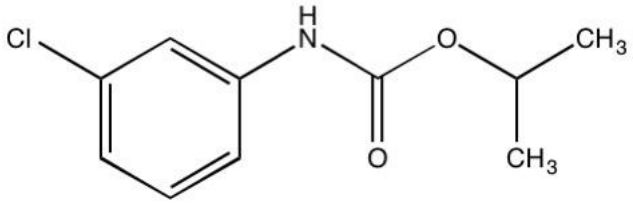


**Figure 29. Chlorpropham (CIPC) structure.**

CIPC is known to inhibit sprouting by altering the structure and function of microtubules and therefore inhibiting cell division (Campbell, Gleichsner et al. 2010). The effects of CIPC on abscisic acid (ABA) content and gene expression in tuber meristems was studied by Campbell *et al.* (2010), and compared to both non-dormant and dormant meristems. As has been discussed, ABA plays a crucial role in the initiation and maintenance of dormancy, and therefore it was hypothesised that CIPC application may disrupt the role that ABA plays during the dormancy period. ABA levels decrease dramatically during the dormancy progression, and CIPC-treated meristems were shown to have the same levels of ABA as the non-dormant meristem control. This suggests that the repression of sprouting achieved by the application of CIPC is not due to an increase in ABA levels (Campbell, Gleichsner et al. 2010). qRT-PCR was also used in order to compare relative gene expression, however significant differences between the transcript profiles of CIPC-treated tuber meristems and the transcript profiles of tuber meristems in the dormant state. This transcriptomic analysis suggests that the mechanism of action of CIPC is not due to an extension of the normal dormant state (Campbell, Gleichsner et al. 2010).

Despite the lack of understanding of the specific mechanism of action of CIPC, it has been used for many years by farmers in the potato industry as an effective method for controlling sprouting due to the fact that its chemical synthesis is inexpensive on a large scale. CIPC is applied to storage barns at several intervals throughout the year as a 'hot fog', during which period CIPC is allowed to circulate throughout the storage unit. For these reasons, CIPC is an attractive sprouting inhibitor, as it can be purchased relatively cheaply by the farmer and applied easily on a large scale when necessary. Potato farmers are therefore highly dependent on CIPC as a means of preventing their crop from premature sprouting.

Other chemicals, such as 1,4-dimethylnapthalene (DMN) and ethylene have also been shown to have sprouting inhibitory properties, however CIPC appears to be the preferred method for prevention due to its cost, ease of application, effectiveness and low impact on processing

quality. In 2013-2104, 1.2 million tonnes of stored potato crop received CIPC treatment – no other viable alternative sprouting suppressant is available for this scale (Compliant 2013).

### 4.1.7 EU Legislation Changes

In recent years, EU legislation has changed in order to regulate the chemicals that are applied to crops destined for the food market, due to concerns over their effects on human health.

Regulations are dependent on the destination of the potatoes that are to be treated but regulations for 2015-2016 include a maximum application of CIPC of 30 g/tonne on fresh market potatoes, and 50 g/tonne on processing market potatoes. In comparison to 2013-2014 regulations, the maximum amount has been decreased (2013-2014 maximum application of CIPC: 36 g/tonne fresh market, 63.75 g/tonne processing market) (Compliant 2013). The last time of application of CIPC is 14 days before removal of potatoes from the store for sale or processing.

Concerns over the toxicity of CIPC to human health stem from mammalian studies in which CIPC affected development or showed hemotoxicity. Tanaka *et al*. (1997) administered CIPC to pregnant mice between days 8 and 11 of gestation at a level of 3000 mg/kg body weight, and the mice were killed at day 18. External malformations of foetuses were observed in all treatment groups (Tanaka, Fujitani et al. 1997). Further studies involved including different concentrations of CIPC in the diet of male and female rats for 13 weeks. Rats fed 30000 ppm CIPC in the diet showed less weight gain than other groups. In addition, red blood cell count, haemoglobin concentration and platelet count were also decreased in rats in the treatment groups (Fujitani, Tada et al. 1997). This study concluded that the erythrocyte is one of the primary targets of CIPC toxicity in rats (Fujitani, Tada et al. 1997). Despite these apparent toxic effects to mammals, it is important to note the levels at which CIPC may be detected on the potato. Current regulations allow a Maximum Residue Level (MRL) of 10 mg/kg CIPC (CIPC Compliant); and therefore it is highly unlikely that a high enough concentration to see the toxic effects of CIPC would be consumed purely by eating CIPC-treated potatoes.

Nevertheless, the EU has imposed strict regulations limiting the application of CIPC to potato crops in storage in order to prevent any detrimental side effects. Maximum application quantities have already been decreased, as previously discussed, however there is speculation within the potato growing industry that maximum allowable CIPC quantities will be reduced further, and may even be withdrawn from the industry completely (Hewitt 2015).

Such regulation by the EU on chemicals to be used on food crops is not just limited to the potato industry. Other examples include the high profile case of neonicotinoid withdrawal as an insecticide for oil seed rape crops due to the potential lethal effects on other insects such as bees. Methyl bromide has also been removed in the U.S, which strawberry growers use as a soil fumigant. A similar situation to the crisis faced by potato growers is faced by strawberry growers as the modern industry of strawberry growing is dependent on the use of methyl bromide, and with no feasible alternative solutions at present, the annual strawberry yield may be drastically affected in future years (Bomgardner 2015).

### 4.1.8 Role of Monoterpenes in Germination Inhibition

CIPC is the most commonly used method for inhibiting sprouting in storage (Kleinkopf, Oberg et al. 2003), however there are other compounds that have also been shown to have sprout inhibiting properties. These include essential oils from plants such as caraway, peppermint and clove, and their components, including *S*-Carvone and eugenol. Despite the fact that these natural products inhibit potato sprouting, their specific targets for inhibition may be different and involve hormone signalling pathways (Rentzsch, Podzimska et al. 2012).

Such secondary metabolites may be released by plants in order to provide a competitive advantage over other plants in the same environment. If the secondary metabolite released by one plant prevents the sprouting or germination of another plant in the vicinity, this increases the chances of survival of the plant releasing the metabolite, therefore giving it a competitive advantage. The release of such secondary metabolites is ecologically beneficial to the plants releasing them. These properties may be exploited in agriculture in order to improve the growth of a particular crop; in this case, plant extracts such as *S*-Carvone can be used to control the germination or sprouting behaviour of crop plants.

The monoterpene *S*-Carvone has been shown to have an inhibitory effect on potato sprout growth and this appears to be due to an interaction of the monoterpene with GA-mediated release of potato bud dormancy. This is thought to be associated with the tissue-specific regulation of α- and β-amylases (Rentzsch, Podzimska et al. 2012). As has been previously discussed, GAs are believed to play a role in dormancy break and the onset of sprouting, and therefore, if *S*-Carvone interferes with the process, sprouting onset may be inhibited.

Due to the fact that monoterpenes, such as *S*-Carvone are produced by plants, it may be interesting to determine whether the same sprouting inhibition property is observed in a crude plant extract as is observed in purified *S*-Carvone.

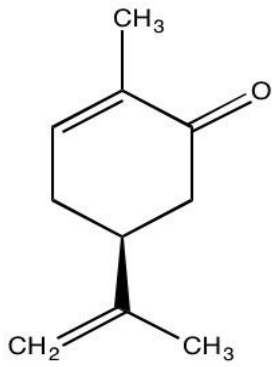


**Figure 30. *S*-Carvone structure.**

As monoterpenes such as *S*-Carvone are natural products extracted from plants, they are attractive candidates for replacing CIPC as a sprout inhibitor for the potato storage industry. However, it should also be noted that essential oils, from plants such as caraway, peppermint and clove are accompanied by distinctive smells. *S*-Carvone also has a distinctive mint-like smell, and therefore there is concern that the use of a compound of this sort may negatively impact on the taste quality of processed potatoes. If this is the case, sprout inhibitors such as this one may not be appropriate for this industry.

Although *S*-Carvone has been identified as having sprouting inhibitor properties, one reason for it not being as commonly used as CIPC may be related to the fact that it may need to be applied on a more regular basis in comparison to CIPC (Kleinkopf, Oberg et al. 2003) due to its volatility and the fact that it is unstable. Therefore, a method for reducing the volatility and increasing the stability of *S*-Carvone may mean that it would not need to be applied on as regular a basis, thereby reducing costs to farmers. One method of improving these properties may be by forming a complex with cyclodextrins.

Cyclodextrins are cyclic carbohydrates which are derived from starch. Cyclodextrins have a hollow, truncated cone structure which is considered to be a 'hydrophobic cavity' due to the fact that polar groups are located on the exterior of the cone structure. This means that the interior of the cavity has a lower polarity than the exterior of the structure, and hence means

that cyclodextrins can encapsulate molecules which would otherwise be insoluble in water (Marques 2010). Furthermore, cyclodextrins are approved as safe as food products by the World Health Organisation, Food and Agriculture Organisation of the United Nations, and by the European Union (Marques 2010). Encapsulation in cyclodextrins may not only provide a useful method for reducing volatility of *S*-Carvone, but may also increase the water solubility and bioavailability of CIPC, meaning that reduced amounts are required for the same sprouting inhibition effect.

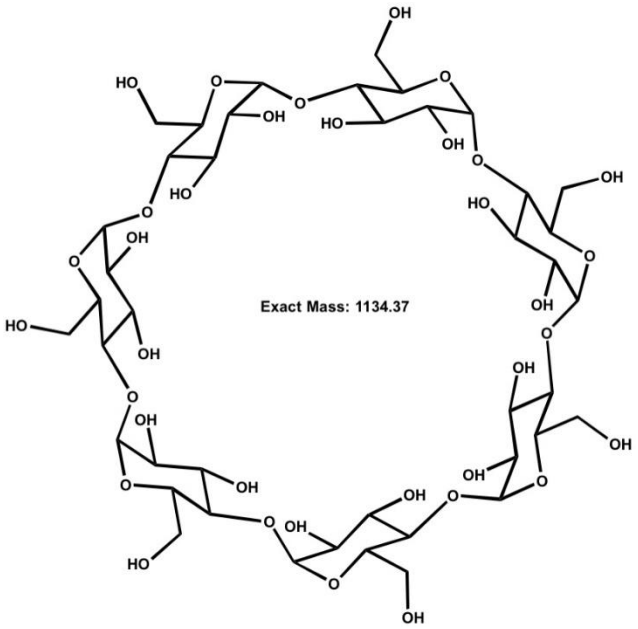


**Figure 31. Structure of β-Cyclodextrin.**

Costa E Silva *et al*. (2007) carried out a study in which *S*-Carvone was complexed with β-cyclodextrin, and this formulation was tested for its sprouting inhibitory effect on potatoes. This study suggested that *S*-Carvone in complex with β-cyclodextrin decreased the level of sprouting more than CIPC (Silva, Galhano et al. 2007), however this study was only carried out over a period of eight weeks as opposed to throughout the storage season.

CIPC has also been complexed with cyclodextrins (hydroxypropyl-β-cyclodextrin (HPBCD)) and the effect of this was also observed in potato sprouting inhibition. Equal levels of potato sprouting were observed in the CIPC control treatment as the CIPC in complex with HPBCD, although the quantity of CIPC applied was more than a ten-fold lower quantity in the CIPC complexed with HPBCD treatment compared with CIPC alone (Huang, Tian et al. 2014). However, this study was carried out on a relatively small scale (20 tubers per treatment) and were stored at 15 $^{o}$C, which is a higher temperature than potatoes would be stored at during industrial potato storage.

### 4.1.9 **Aims**

The aim of this study is to determine the effect of the monoterpene, *S*-Carvone, on potato sprouting over the period of normal industrial potato storage (October-July). Cyclodextrins will also be used to determine their effect on efficacy of sprouting inhibitor. Along with observational analysis with regards to level of sprouting, data will be collected in order to determine the effect of these chemical treatments on processing quality of the potato after it has been stored with these treatments.

This is the first study of its kind to observe the processing quality of tubers following treatment with these alternative sprouting inhibitors, particularly on a scale that largely reflects industrial potato storage.

## 4.2 Results

### 4.2.1 Farm Trial Results

Visible sprouts were first recorded on 04.12.2014 and therefore figures 32 - 33 show the difference in average sprout number between the date that visible sprouts were first observed and the final date of observation (30.06.2015). In all cases, an increase in average sprout number was observed, as would be expected of potato tubers approximately nine months after going into store. Comparison of data sets suggests the extent to which potatoes sprout was highly dependent on variety, as variable patterns were observed between varieties, despite receiving the same treatments and being stored in the same conditions.

Following application of treatment compounds, sprout length and sprout number were recorded on a weekly basis. The data displayed here in bar charts compares the differences in sprouting between dates at the start and end of the trial. Some data sets did not follow the assumptions required for parametric ANOVA testing, including failing to fit a Normal distribution, and not having equal variance. Therefore, data was statistically analysed using the non-parametric equivalent of ANOVA, known as Kruskal-Wallis testing. Mann-Whitney U tests were used for post-hoc analysis of data to determine statistical differences between groups. Statistical comparisons were made against potatoes treated with the conventional, in-store CIPC treatment in order to determine whether the trial compounds being tested showed similar levels of sprouting inhibition.

### 4.2.2 Statistical hypotheses

As the Kruskal-Wallis and Mann Whitney U tests are non-parametric tests, they analyse data based on rank. Therefore, the null hypothesis in the case of this study declared that two compared treatment groups come from the same population and did not show differences based on rank (ie. $P(X>Y) = P(Y>X)$). The significance value used was $p < 0.05$.

There appeared to be few differences between average sprout number at the beginning of the trial (4/12/14) when comparing treatment groups to the CIPC control (Figures 32, 33). This may be explained by the fact that tubers may still be within the period of dormancy, and therefore all tubers exhibited a low level of sprouting regardless of their treatment. More trial groups appeared to be significantly different compared to the CIPC control at this time point (4/12/14), when considering sprout length data. This suggested that sprout length was more affected by sprouting inhibitor treatment early on in the storage period than sprout number was. Of the

three varieties, Hermes showed the greatest number of groups differing significantly from the control, which supported the fact that Hermes is known to have a shorter dormancy period than the other varieties observed.

At the end of the trial (30/06/15 - eight months after the initial treatment application), significant differences were observed between the control and many of the treatments for both sprout number and sprout length data sets. Crucially, it should be noted that the aqueous application of CIPC, used as a control to account for differences in application, was significantly different from the conventional method of applying CIPC. Therefore, it may be concluded that method of application impacted on the efficiency of the treatment applied.

Some treatment groups did not show significant differences when compared to the CIPC control, however consistency was lacking between varieties and was dependent on the observation being made. CIPC x HPBCD treatment was an example of this: Lady Claire sprout number was not significantly different from the control (Figure 32 – 2), whereas sprout length was significantly different (Figure 34 – 2). In contrast, both Hermes and Markies did not show significant differences between this treatment and the control when observing sprout length (Figure 34 – 1,3), but did show significantly different sprout numbers (Figure 32 – 1,3). Therefore, it may be concluded that response to the sprouting inhibitor treatment was variety dependent.

Differences were also observed when looking at Hermes data for *S*-Carvone treatment. Sprout number appeared to show no significant difference compared to the control, whereas sprout length is significantly different. This highlights the fact that sprouting observations with individual parameters, such as length or number, were not a good indicator of the extent to which a potato was sprouted.

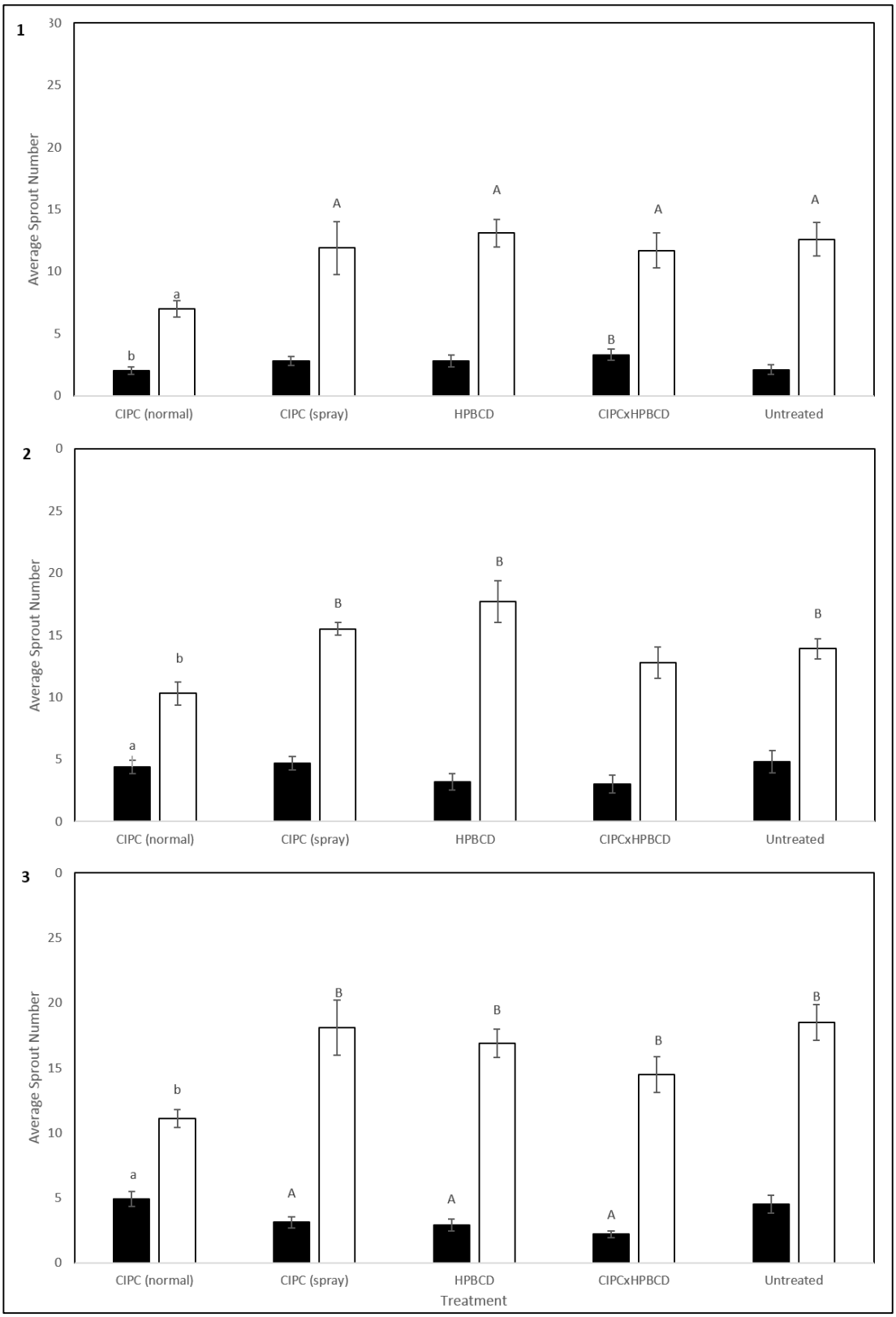


**Figure 32. Average sprout number per tuber - CIPC.**

Average sprout number per tuber in different varieties when treated with CIPC trial compounds. Varieties (1) Markies, (2) Lady Claire, (3) Hermes.
(▪(black): 04/12/2015, ▫(white): 30/06/2015). Results are expressed as mean ± SE, *n* = 10. Mann-Whitney U test: $p<0.05$. Statistical testing within data sets, against lower case group (a/b), significant result ($p<0.05$) reported as upper case (A/B).

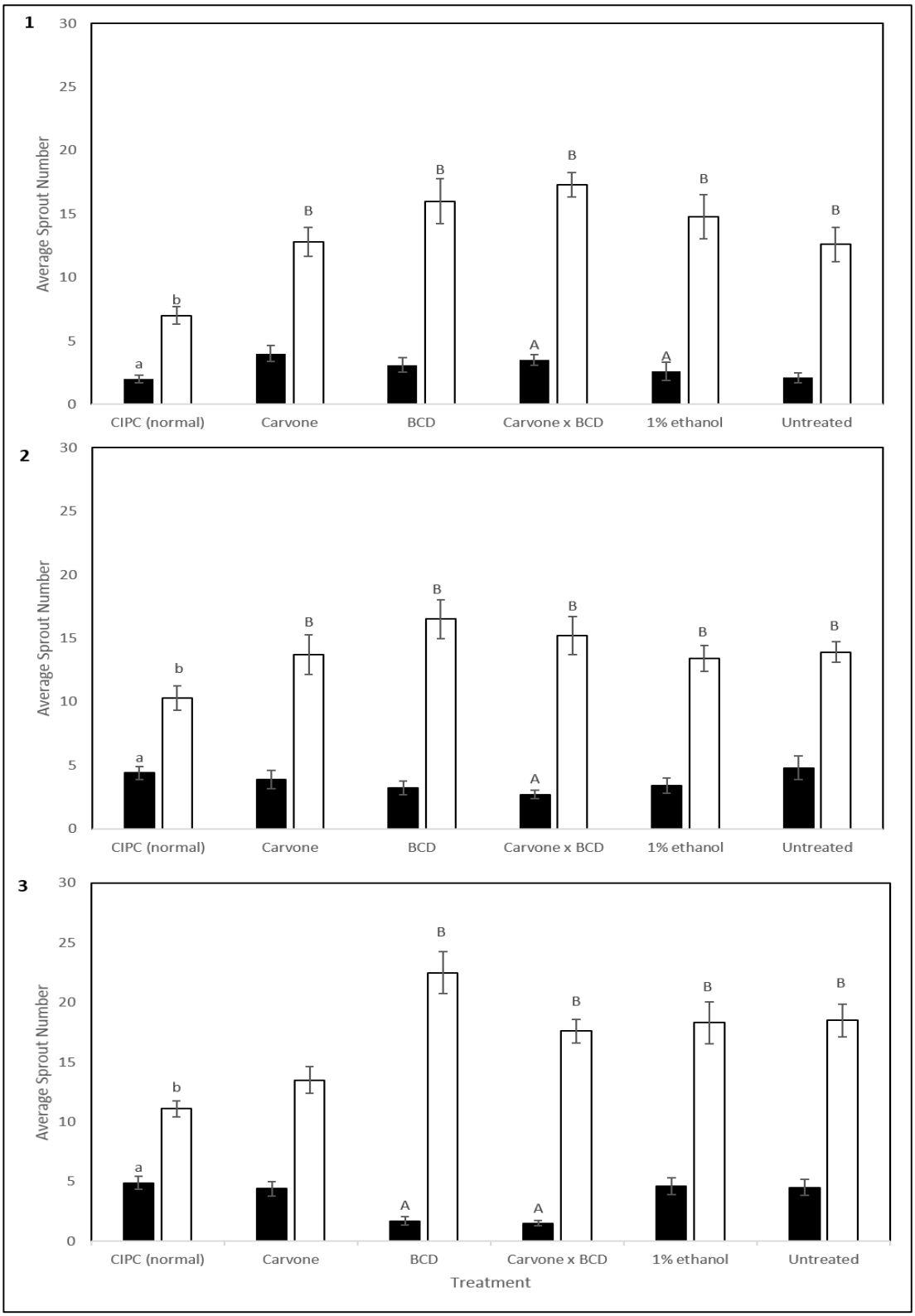


**Figure 33. Average sprout number per tuber - *S*-Carvone.**

Average sprout number per tuber in different varieties when treated with *S*-Carvone trial compounds. Varieties (1) Markies, (2) Lady Claire, (3) Hermes.

(▪(black): 04/12/2015, ▫(white): 30/06/2015). Results are expressed as mean ± SE, *n* = 10.

Mann-Whitney U test: $p<0.05$. Statistical testing within data sets, against lower case group (a/b), significant result ($p<0.05$) reported as upper case (A/B).

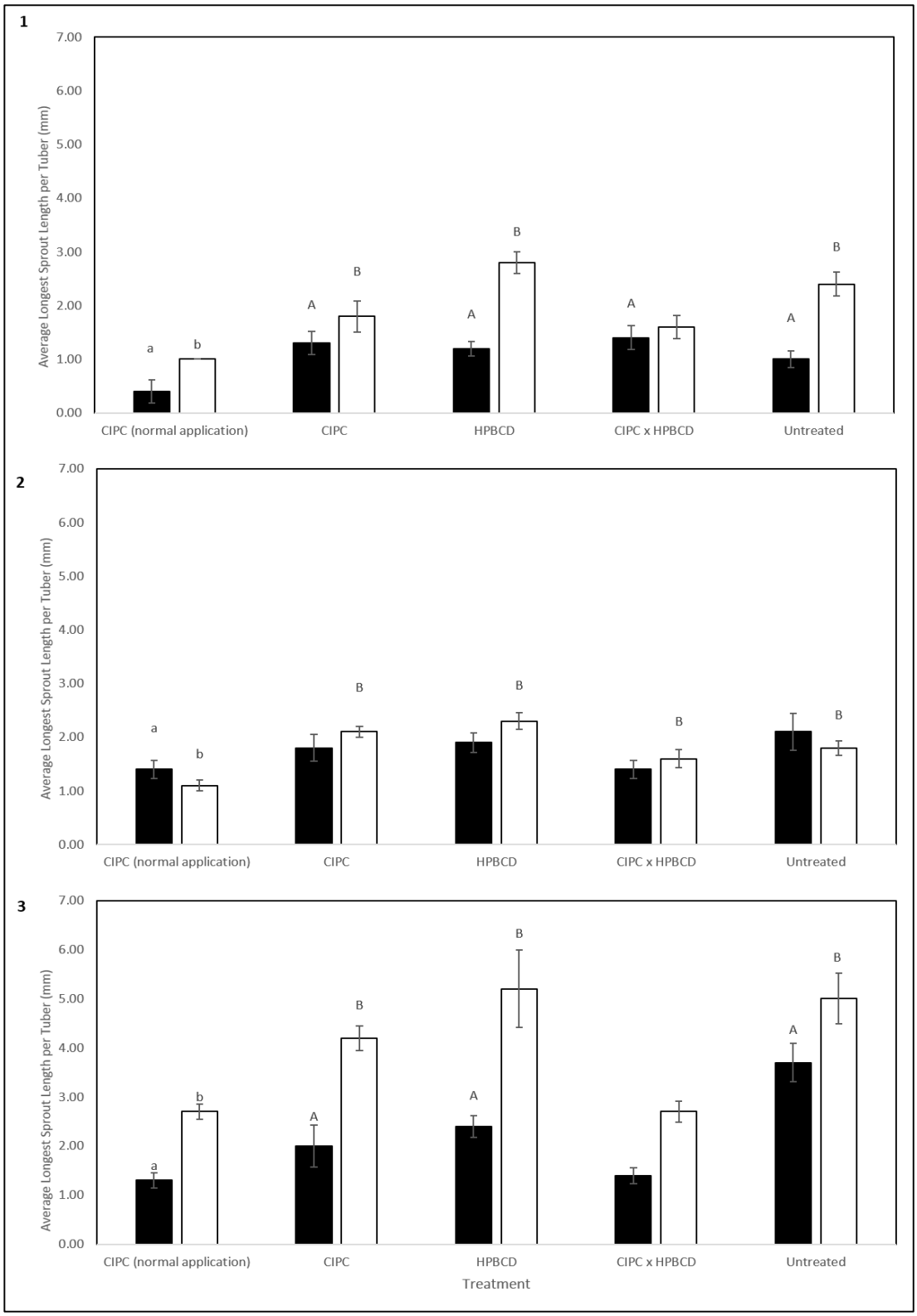


**Figure 34. Average longest sprout length per tuber - CIPC.**

Average longest sprout length per tuber in different varieties when treated with CIPC trial compounds. Varieties (1) Markies, (2) Lady Claire, (3) Hermes.

(▪(black): 04/12/2015, ▫(white): 30/06/2015). Results are expressed as mean ± SE, *n* = 10.

Mann-Whitney U test: $p<0.05$. Statistical testing within data sets, against lower case group (a/b), significant result ($p<0.05$) reported as upper case (A/B).

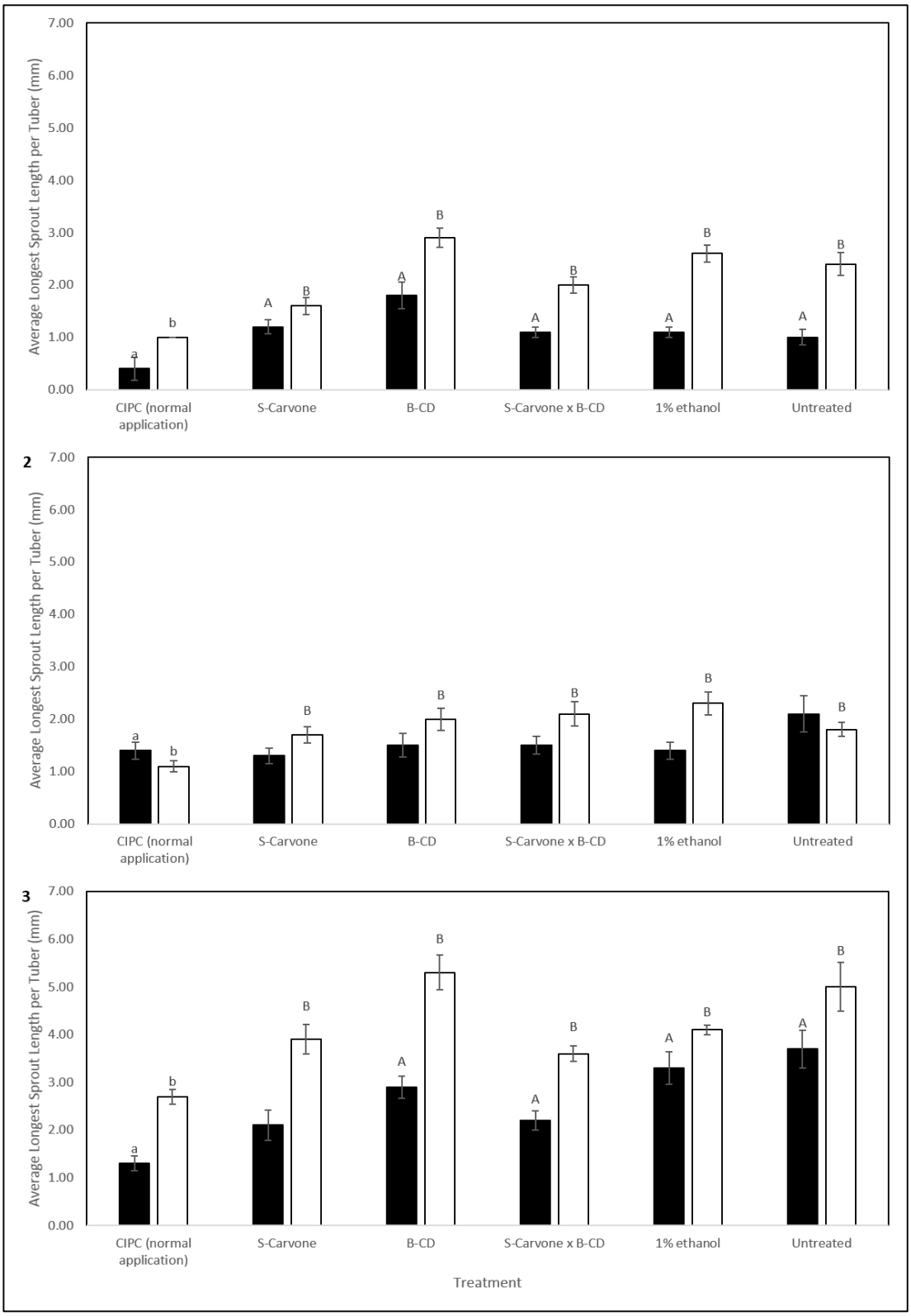


**Figure 35. Average longest sprout length per tuber - *S*-Carvone.**

Average longest sprout length per tuber in different varieties when treated with *S*-Carvone trial compounds. Varieties (1) Markies, (2) Lady Claire, (3) Hermes.

(▪(black): 04/12/2015, ▫(white): 30/06/2015). Results are expressed as mean ± SE, *n* = 10.

Mann-Whitney U test: $p<0.05$. Statistical testing within data sets, against lower case group (a/b), significant result ($p<0.05$) reported as upper case (A/B).

### 4.2.3 Processing Quality of Potatoes

Any chemical treatments to be used on crops must not impact on the quality of the end product. In the case of potatoes grown for crisping, chemicals must not detrimentally affect the processing quality of the potato. As discussed, the presence of excess sucrose in the tuber can lead to darkening of the processed product, and therefore it was important to ensure that the sprouting inhibitor compounds being applied did not affect the colour of the crisp.

Frying was carried out at Nelson County Potatoes Ltd (Gimingham, North Norfolk) by the Quality Control team. In order to ensure potatoes met the standard demanded by the industry, the fried product was compared to the Potato Council Quality Control. Potatoes were also homogenised and assayed using the YSI 2950 Biochemistry Analyser to test for sucrose concentrations.

Potato crisp colours were acceptable when compared to the colour charts, meaning that they would be accepted by processors (Figures 36 - 37). Sucrose concentration was also found to be below the upper limit for processing (data not shown), and therefore the tubers would be accepted for processing based on this data.

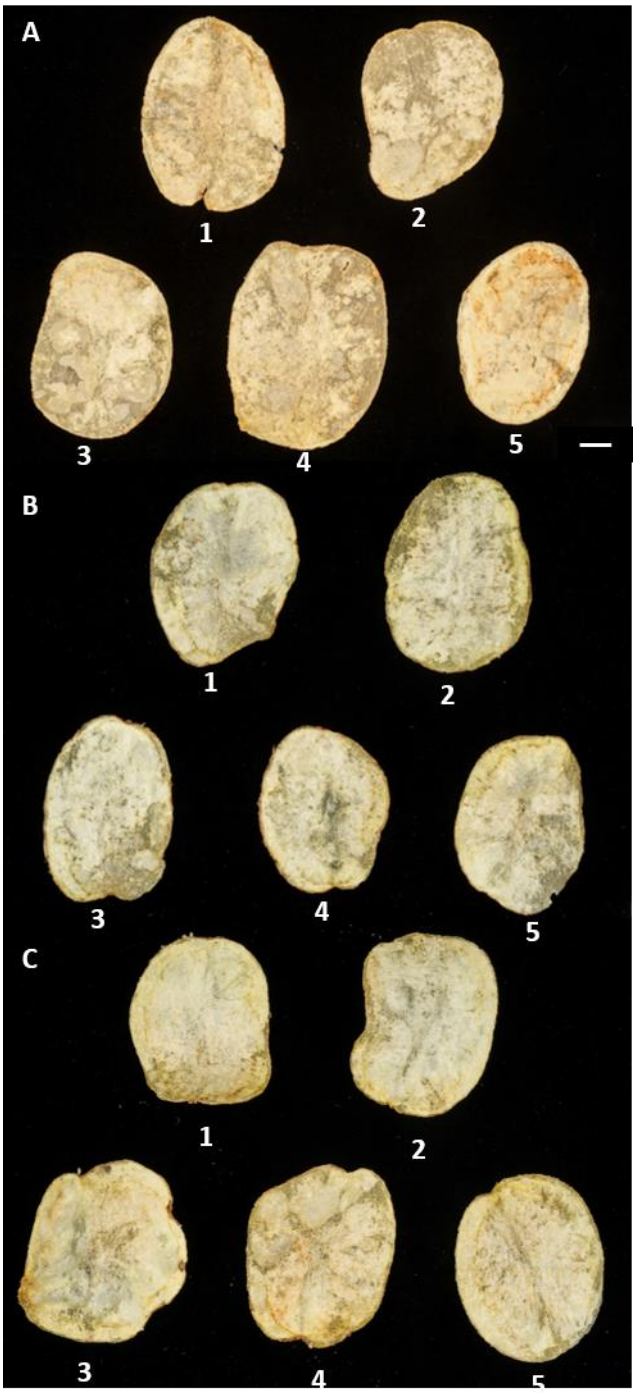


**Figure 36. Potato fry colours - CIPC.**

Potatoes processed into crisps to determine the effect of chemical treatments on processing quality. Varieties: (A) Markies; (B) Lady Claire; (C) Hermes. Treatments: (1) Untreated; (2) CIPC normal – conventional treatment; (3) CIPC aqueous spray; (4) HPBCD; (5) CIPC x HPBCD.
Scale Bar = 1 cm.
Crisps compared to the Potato Council fry quality colour chart (Appendix 3).

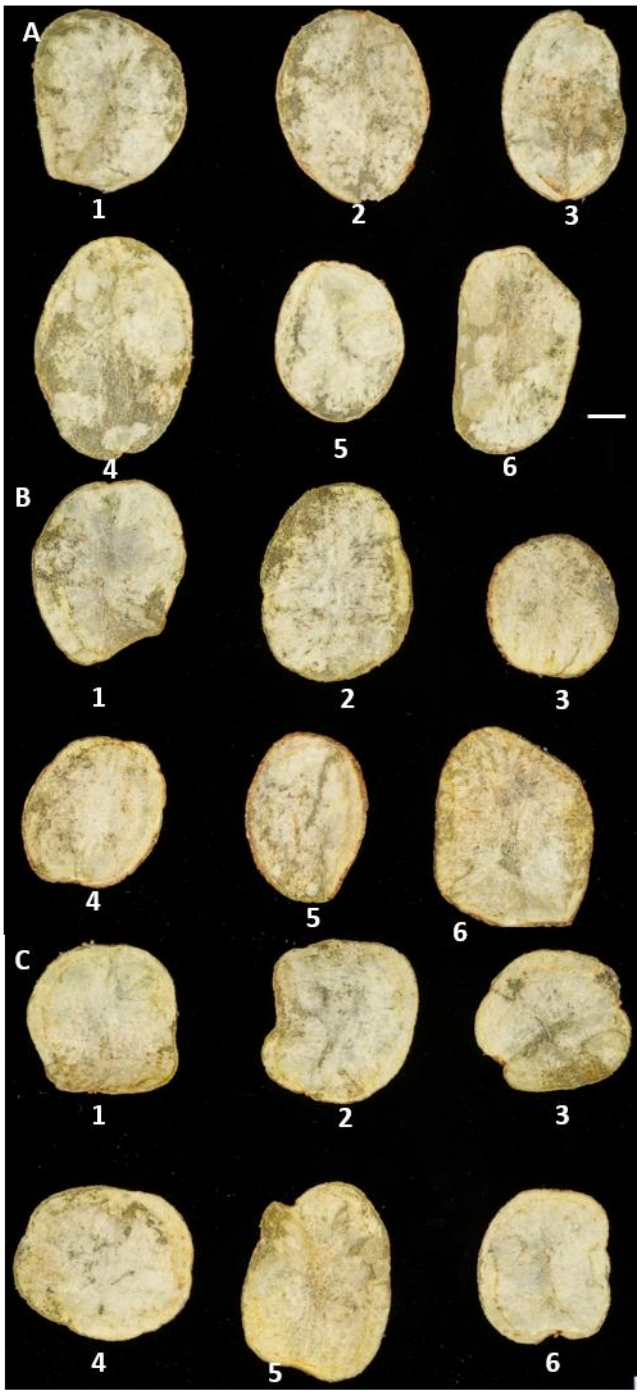


**Figure 37. Potato fry colours - *S*-Carvone.**

Potatoes processed into crisps to determine the effect of chemical treatments on processing quality. Varieties: (A) Markies; (B) Lady Claire; (C) Hermes. Treatments: (1) Untreated; (2) CIPC normal – conventional treatment; (3) *S*-Carvone; (4) BCD; (5) *S*-Carvone x BCD.
Scale Bar = 1 cm.
Crisps compared to the Potato Council fry quality colour chart. (Appendix 3).

### 4.2.4 **Compound Residue Analysis**

Following completion of sprouting observations, tubers were peeled and methanol was used to extract the treatment compounds from the potato peel samples. Extracts were analysed by Liquid Chromatography – UV (LC-UV) against known concentrations of chemical standards. Liquid Chromatography – Mass Spectrometry (LC-MS) was used, however it appeared as though neither CIPC nor *S*-Carvone could be ionised by electrospray in the concentrations that they were present in. For this reason, UV was used as a detection method, with a wavelength of 238 nm observed. Comparison of absorbance peaks of chemical standards with samples provided evidence that the peaks observed in the samples were CIPC and *S*-Carvone, as absorbance peaks were observed at the same retention time (Figure 38). However, as mass data was not available, this evidence was not definitive.

Regardless of this, an attempt was made to quantify the concentration of compound residue in each sample. Comparison of CIPC concentration in the methanol extracts was different between conventionally- and aqueous spray-treated, despite the fact that CIPC was applied in the same concentration for each sample (12 g/tonne). This further supports the hypothesis that application method significantly affected the efficiency of sprouting inhibitor treatment, as this data suggested that the conventional hot fog application method resulted in a higher concentration of CIPC being present on the potato peel at the end of the trial when compared to aqueous application of CIPC.

Peaks were detected at the wavelength of 238 nm, which corresponded to the CIPC and *S*-Carvone standards in all samples (Figure 38). This detection may have been due to other compounds absorbing the UV wavelength at 238 nm, therefore explaining why peaks were observed in all samples. CIPC was detected in higher concentrations than *S*-Carvone, however relative levels of the compounds correlated with the treatment to which tubers were exposed (ie. Higher levels of *S*-Carvone were detected in extracts treated with *S*-Carvone compared to extracts from potatoes treated with other compounds) (Figure 39). Alternative techniques, along with LC-UV were used to confirm the presence of CIPC and *S*-Carvone: Thin Layer Chromatography, GC-MS, and Accurate Mass were carried out (data not shown), however results were still not definitive following this analysis.

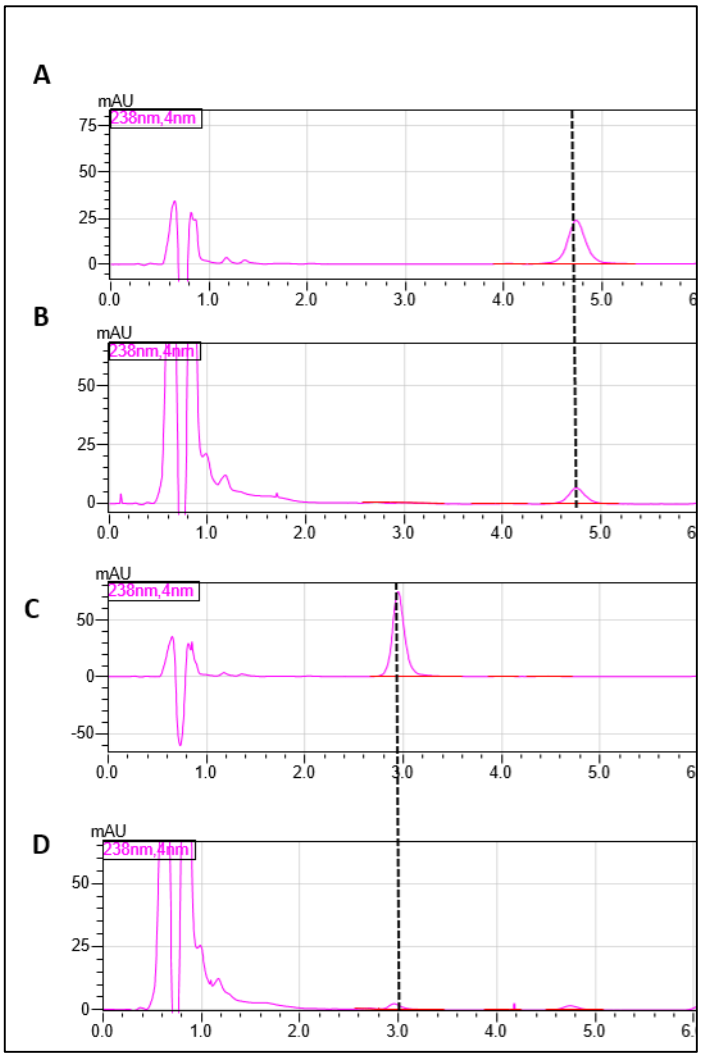


**Figure 38. LC-UV spectra of methanol extracts from potato peel samples.**

LC-UV used to detect CIPC and *S*-Carvone in potato peel methanol extracts.
(A) CIPC standard (0.01 mg/ml); (B) Markies CIPC normal; (C) *S*-Carvone standard (0.01 mg/ml); (D) Markies *S*-Carvone. UV wavelength of 238 nm used to detect absorbance. Dashed line represents co-eluting peaks.

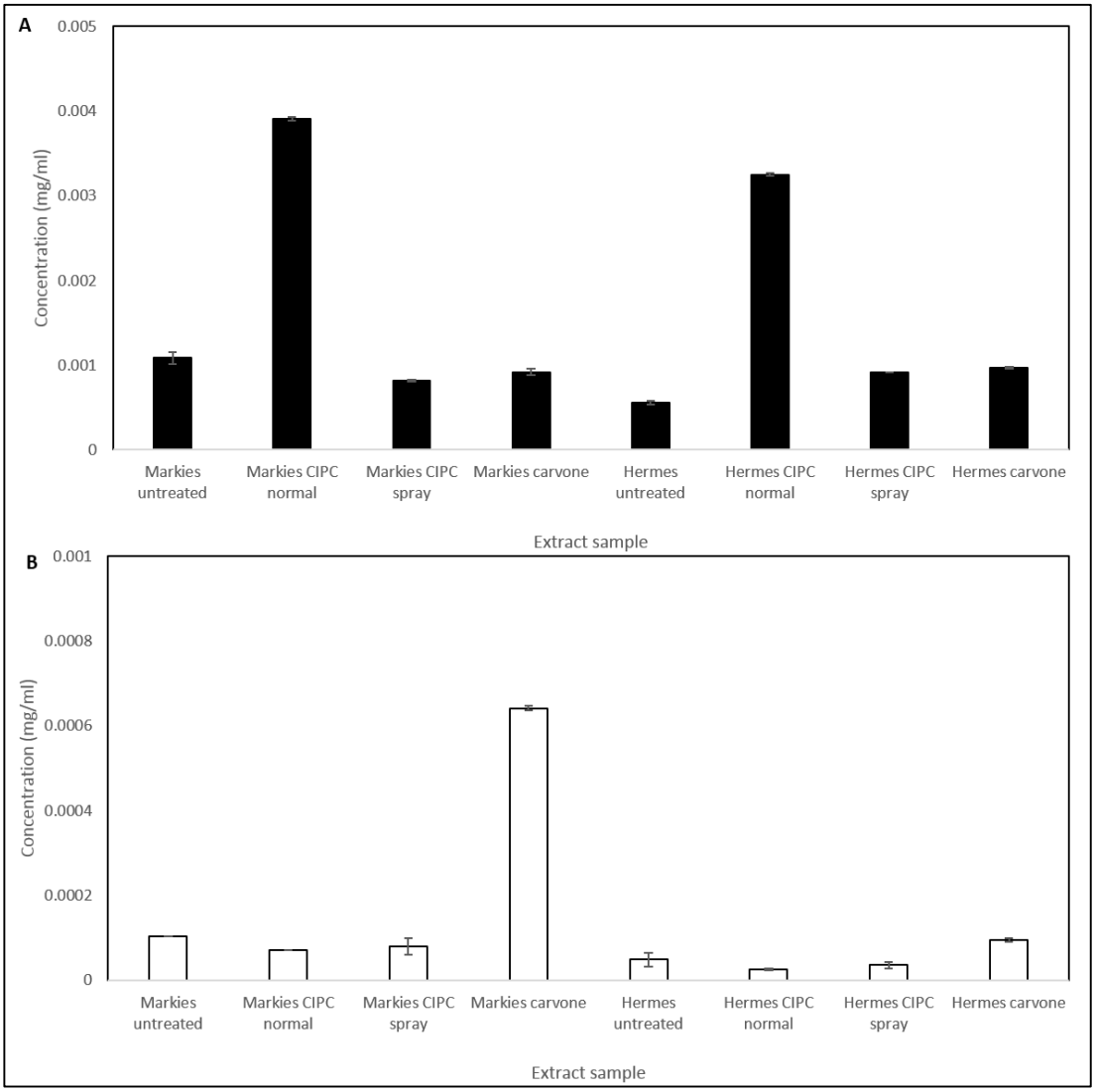


**Figure 39. LC-UV analysis of CIPC and *S*-Carvone concentrations.**

Concentration of (A) CIPC, and (B) *S*-Carvone in trial sample methanol extracts. Concentration calculated following LC-UV analysis and comparison with calibration curve for the two compounds.
Results expressed as mean ± SE, n=2.

### 4.2.5 *S*-Carvone concentration box trial

As *S*-Carvone was potentially detected via LC-UV in samples other than the ones treated with this compound, it may be possible that the volatile nature of *S*-Carvone allowed it to circulate in the atmosphere. Therefore it was hypothesised that *S*-Carvone may play a role in suppressing sprouting in potatoes other than those directly treated with the *S*-Carvone aqueous spray. A secondary trial experiment was established whereby the effect of atmospheric volatile *S*-Carvone was tested both at 9 °C and at 22 °C.

Average sprout number and average longest sprout length per tuber (mm) were recorded at the start and end of the trial (8 weeks later). Results were statistically compared to the untreated control (Box 1). A greater number of significant differences were observed in the trial stored at 9 °C compared to the trial stored at 22 °C. It appeared that temperature played a significant role in the extent to which potatoes sprouted, as the data was not consistent between the two trials, despite the fact that both trials received the same treatments. The presence of *S*-Carvone in the 9 °C trial had a concentration-dependent effect on sprout number (Figure 40A), in which sprout number decreased with increasing *S*-Carvone concentration. Sprout length did not show this concentration-dependent response, although the sprout length of tubers in the highest concentration box did show a significant difference when compared to the control (Figure 40B).

Results from the 22 °C trial were not consistent with those from the 9 °C trial (Figure 41). *S*-Carvone appeared to have less of an effect on both sprout number and sprout length at 22 °C compared to at 9 °C (Figure 42A, B). Sprout number increased drastically at 22 °C compared to at 9 °C (Figure 42). This may indicate that *S*-Carvone was able to inhibit sprouting at lower temperatures, when sprouting may still have been partially suppressed by dormancy, but was not able to inhibit sprouting when the temperature was high enough to promote sprouting. The data indicates that *S*-Carvone was able to inhibit sprouting to some extent, although was not potent enough alone to prevent sprout development when other environmental factors promoted this.

Again, the trends in sprout number and sprout length did not always correlate (Figures 40, 41).

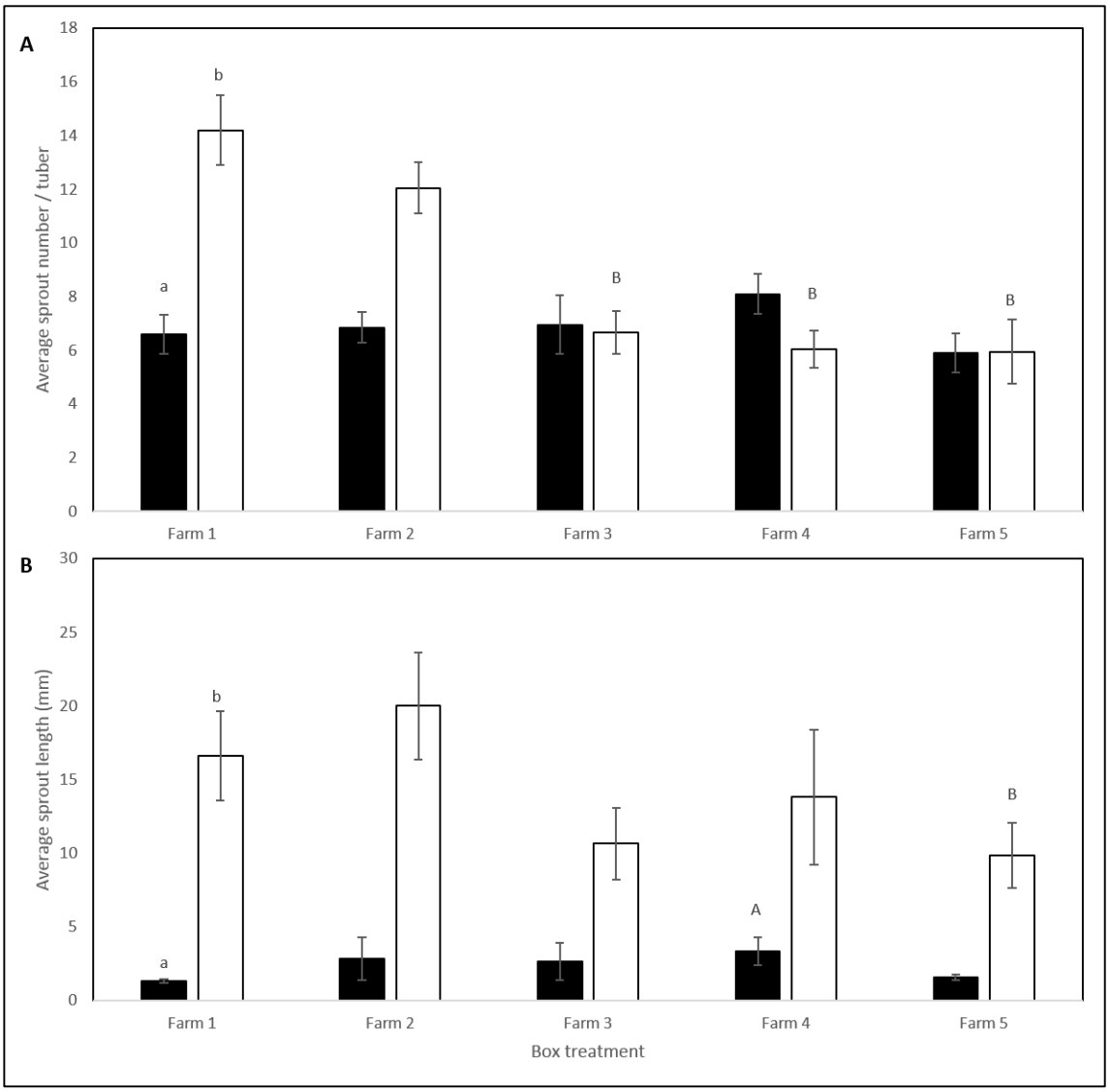


**Figure 40. Sealed box *S*-Carvone trial data - Farm.**

Average sprout number per tuber and average sprout length of tubers treated with varying concentrations of *S*-Carvone and stored within sealed boxes. Boxes stored at 9 °C throughout trial.

(■(black bars): 22/05/2015, □(white bars): 17/07/2015). Results expressed as mean ± SE, n = 20.

Mann-Whitney U test: $p<0.05$. Statistical testing within data sets, against lower case group (a/b), significant result ($p<0.05$) reported as upper case (A/B).

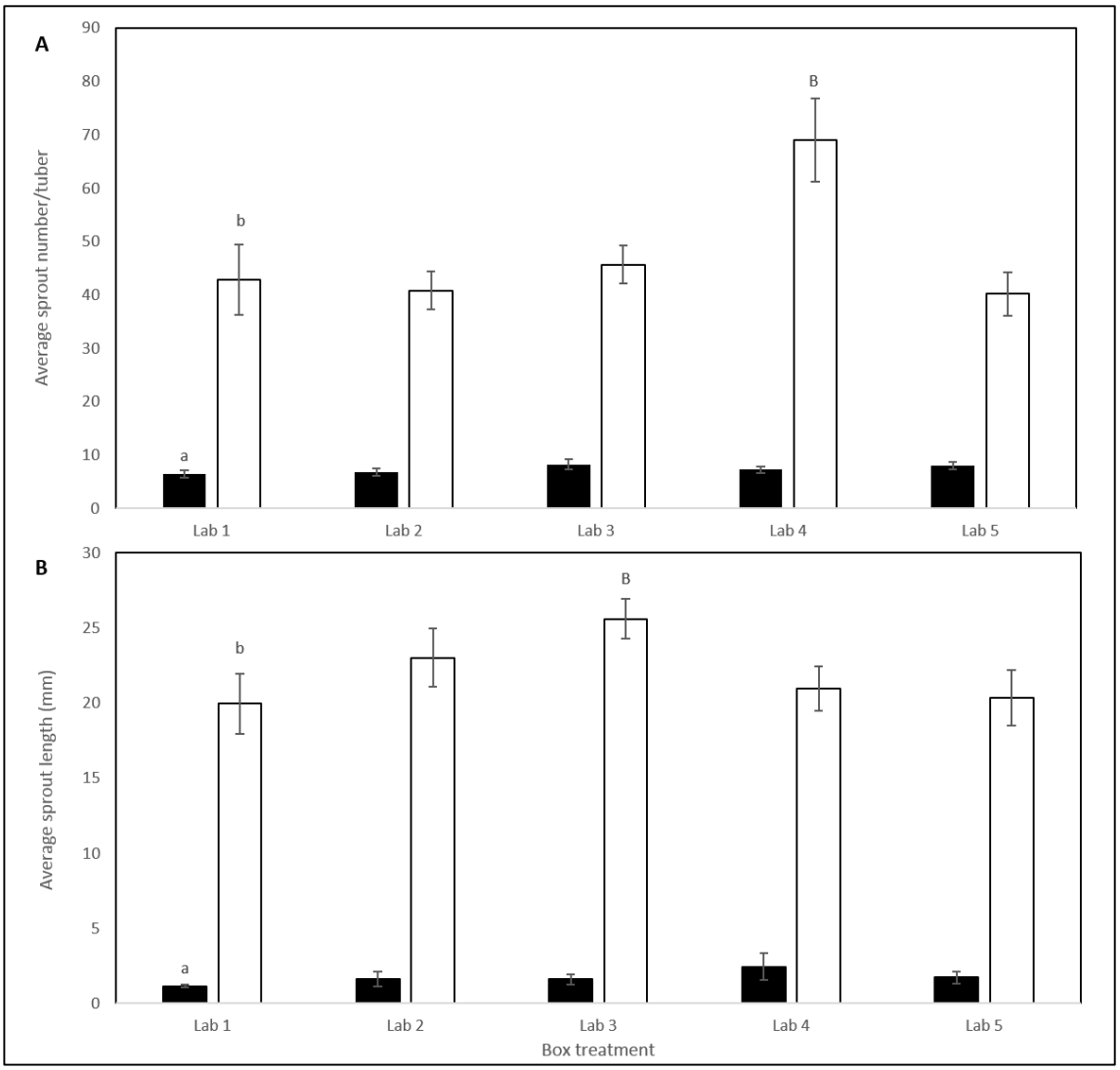


**Figure 41. Sealed box *S*-Carvone trial data -Lab.**

Average sprout number per tuber and average sprout length of tubers treated with varying concentrations of *S*-Carvone and stored within sealed boxes. Boxes stored at 22 °C throughout trial.

(■ (Black bars) 22/05/2015, □ (White bars) 17/07/2015). Results expressed as mean ± SE, n = 20.

Mann-Whitney U test: $p<0.05$. Statistical testing within data sets, against lower case group (a/b), significant result ($p<0.05$) reported as upper case (A/B).

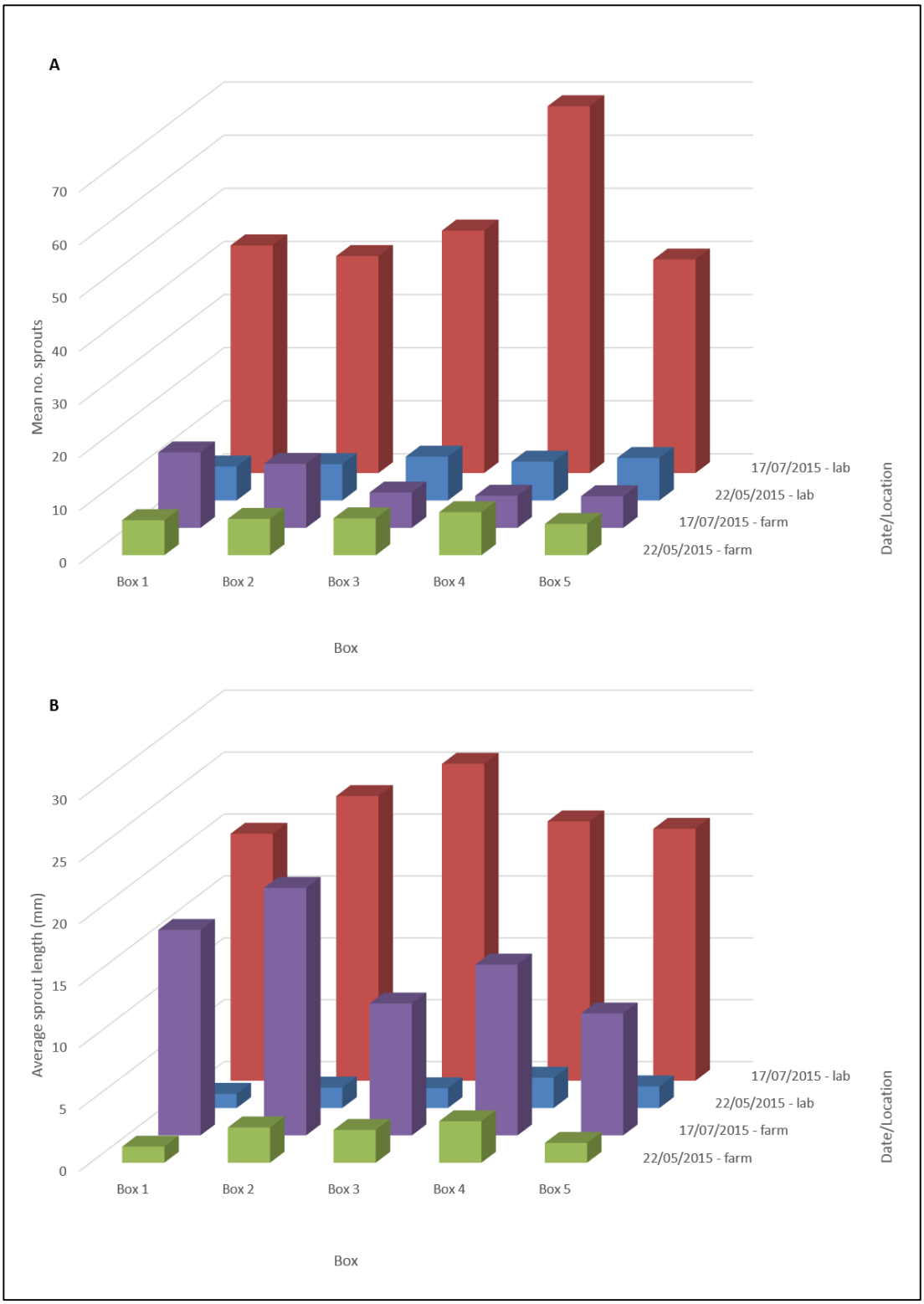


**Figure 42. *S*-Carvone sealed box trial data.**

(A) Average Sprout number per tuber; (B) Average sprout length (mm) of tubers treated with varying concentrations of *S*-Carvone and stored within sealed boxes at either 9 °C (farm), or 22 °C (lab). Results expressed as mean, n = 20.

### 4.2.6 Residue Testing Results

Due to its volatile nature, Solid-Phase Microextraction was used to extract *S*-Carvone released into head-space gases once sprouts were removed from tubers and placed into a sealed round-bottom flask. Gas Chromatography – Mass Spectrometry (GC-MS) analysis was used to determine the nature of compounds present in the head-space gases.

Figure 43 displays the GC-MS chromatographs for head-space gases released from the potatoes stored in sealed boxes at 9 °C and at 22 °C, and extracted using the SPME system. An *S*-Carvone chemical standard was run on the GC-MS to determine the retention time, which was found to be at 9.76 minutes. Peaks corresponding to a retention time of 9.76 minutes were not present in either the blank control, nor in the negative control (Box 1) for either the 9 °C or 22 °C trial (Figures 43 A,B), therefore displaying that *S*-Carvone was not present either on the extraction fibre or endogenously in potato tuber sprouts. Peaks corresponding to a retention time of 9.76 minutes were present in the chromatogram for Box 2, Box 3, Box 4 and Box 5, which were treated with a concentration series of *S*-Carvone. Therefore this provides evidence for the fact that *S*-Carvone was released into head-space gases as a result of being treated with exogenous *S*-Carvone as opposed to potato sprouts releasing an endogenous form of the compound. This was supported by the fact that the mass spectra data of the box trial samples corresponded to the mass spectra of the *S*-Carvone standard, with an m/z ration of 150.1 (Figure 44). Identification of *S*-Carvone was further confirmed by comparison to the NIST 2.0 library.

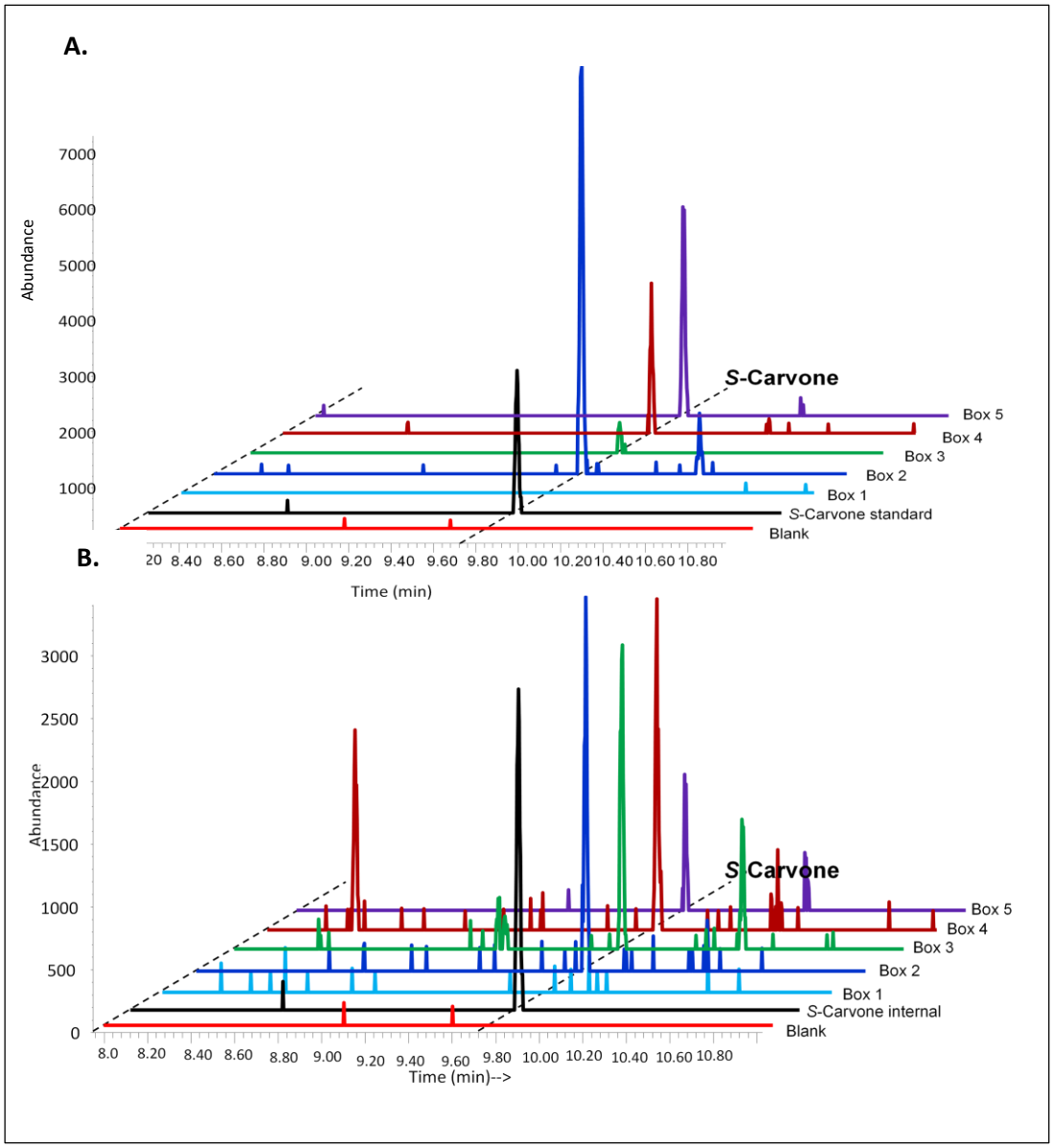


**Figure 43. Headspace gas SPME-GC-MS chromatogram.**

Headspace-SPME-GC-MS extracted ion chromatogram (m/z: 149.80 - 150.80) of headspace gases of potato peel sample stored at (A) 9 °C; (B) 22 °C after treatment with varying *S*-Carvone concentrations. *S*-Carvone retention time at 9.76 minutes.

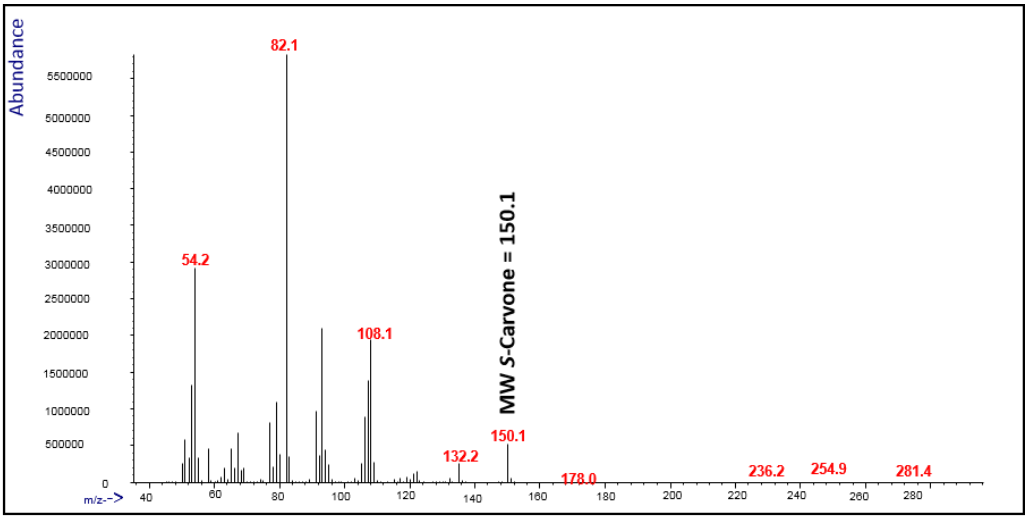


**Figure 44. Electron ionisation (EI) mass spectra of *S*-Carvone.**

*S*-Carvone m/z = 150.1

## 4.3 Discussion

This study has indicated that the application method of CIPC is important in determining how effective the treatment is at inhibiting sprouting. Although trial compounds did not appear to be as effective as the conventional treatment method (CIPC 'fogging'), it should be noted that sprouting in CIPC x HPBCD (CIPC complexed with HPBCD) and *S*-Carvone treated potatoes was comparable with sprouting observed in tubers treated with aqueous CIPC. This control accounts for the error in application method. In order to directly compare the efficacy of CIPC x HPBCD and *S*-Carvone, a further study in which the same method of application is used is necessary.

As the response to the sprouting inhibitor appears to be variety dependent, this supports the fact that potato dormancy and sprouting are complex biological processes with intricate mechanisms of control and regulation, as has been documented in the literature (Sonnewald and Sonnewald 2014). Clearly, a sprouting inhibitor should be universally effective across potato varieties, and this means that further studies should test compounds on more varieties. The data not only shows inconsistences between varieties, but also between sprout length and sprout number. Therefore using a single parameter for quantification of sprouting is inadequate. Although two parameters (sprout length, sprout number) have been used in this study, further work could be carried out to determine a more robust method for sprout quantification.

Regardless of the difficulties presented with a study of this sort, the data does suggest that some of the treatment compounds have sprouting inhibition properties. Importantly, the treatment compounds do not appear to affect the processing quality of potatoes. This is an important variable to consider, as compounds which have major biological consequences such as prolonging dormancy or inhibiting sprouting, may impact on the quality of processed potatoes. Clearly, compounds with such effects are inappropriate for use in the industry, regardless of how effective the compound is at inhibiting sprouting.

Further evidence to support the idea of sprouting being a highly complex biological process is provided by the inconsistencies observed in sprouting when potatoes were treated with different *S*-Carvone concentrations at different temperatures. The data suggests that *S*-Carvone does inhibit sprouting at low (9 $^{o}$C) temperatures, but that it is not capable of inhibiting sprouting at higher temperatures (22 $^{o}$C). Sprouting is clearly controlled by complex regulatory mechanisms. The data in this study suggests that some factors, such as temperature, may have a greater control over the sprouting process than others. Therefore, an array of environmental

factors in combination with sprouting inhibitor compounds must be carefully controlled for optimal sprouting inhibition.

Volatile compounds such as *S*-Carvone are interesting potential sprouting inhibitor treatments for the potato industry, as they are able to circulate in the atmosphere and have an effect on sprouting without being directly applied to potatoes. This would overcome the problem that is currently faced by the potato industry when applying the conventional CIPC treatment with regards to ensuring that all potatoes receive an application of CIPC. Due to the layout of potato storage units, it is difficult to ensure that all tubers receive an equal amount of CIPC, and there is often high variability in residue levels within the same storage units (Personal communication (Hewitt 2015)). Use of a volatile compound which displays inhibitory sprouting properties when in the atmosphere would be beneficial as it would help to mitigate this problem of uneven chemical distribution. The evidence from sealed box trials suggests that *S*-Carvone may inhibit sprouting when in the atmosphere. GC-MS analysis of head-space gases indicates that potato sprouts absorb *S*-Carvone from the atmosphere and release it back into the atmosphere. This, in theory, would provide a more effective method for circulating the compound through the store and ensuring even distribution.

The detection of a compound by LC-UV with the same retention time as CIPC in all potato samples could be due to a background level of absorbance, or may be due to cross-contamination between trials. Samples not directly treated with CIPC absorbed UV at 238 nm; CIPC may have been present in these samples as a result of contamination from storage boxes. As CIPC is not particularly volatile, it is unlikely to be released into the atmosphere in the same way that *S*-Carvone has been shown to. However, as storage units are treated with CIPC, potatoes may have picked residues of CIPC up from these boxes before direct application of any treatment compounds. A method for detection of low concentrations of CIPC would therefore be a useful tool for farmers. If CIPC is withdrawn from the industry, potatoes would need to be stored in boxes not contaminated with CIPC, in order to prevent contamination via the boxes. A method such as the one used in this study, in which methanol was used to extract CIPC may be useful. Although LC-UV detection alone does not unequivocally identify the compound detected as CIPC, the fact that the samples tested correspond to the CIPC chemical standard peak suggests that this is the compound being detected.

Whilst this study has not provided definitive evidence for a potential role of cyclodextrins in sprouting suppression, their use in this industry should be studied further. Other studies of a

similar nature (Silva, Galhano et al. 2007, Huang, Tian et al. 2014) suggest that they do have a role in sprouting suppression and this may be due to complex formation with sprout suppressants. Alternatively, cyclodextrins may impact on other sprouting regulatory processes in the tuber. For example, cyclodextrins may complex with hormones and therefore affect the roles of these hormones during the sprouting process. Further studies would be required to determine whether this is the case.

This study supports the fact that potato sprouting is a highly complex biological process which is affected by many factors. There is evidence to suggest that some of the compounds tested in this study may act as effective sprout inhibitors, although the evidence is inconclusive due to the differences observed as a result of application method. The quantification of sprouting should clearly use a variety of parameters, and the effect of sprouting inhibitors on downstream processing of potatoes should be taken into account. This is the first study of its kind to use processing quality as a measure of how appropriate a sprouting inhibitor is for use in the potato industry.

### 4.3.1 **Future Work**

Work to establish an effective method of application of sprouting inhibitor should be carried out, in order to directly compare the difference between the conventional CIPC 'hot-fog' treatment against alternative sprouting inhibitors. A robust method of determining the extent to which a potato is 'sprouted' could also be developed, as it has been shown that measuring individual parameters is not the most effective or reliable method for measuring tuber sprouting.

Further work may also be carried out to determine the role that cyclodextrins may play in sprouting suppression, and whether this may be related to complexation with sprouting suppressants, or whether they affect other aspects of dormancy and sprouting regulation.

Detection of inhibitor compounds by LC-UV should be confirmed, and this ideally requires mass spectrometry data to confirm their presence. This has not yet been achieved, and therefore extraction and analysis by LC-MS could be further optimised.

# 5 Conclusions

This study has investigated two problems facing potato growers: Potato Cyst Nematode as a pathogen of the potato crop, and the problem of sprouting inhibition whilst the potato crop is in storage. Whilst the study has been relatively broad in scope, several key points have been highlighted, which may be key to improving potato crop productivity, both with regards to mitigating against pathogenic factors, and preventing losses in storage.

The role of terpenes, both as products of the potato, and as compounds used to protect the potato, have been discussed throughout the study, and the conclusions drawn support the statements made by Singh and Sharma (2015) suggesting that the vast array of terpenes may be exploited for their biotechnological purposes. Furthermore, a deeper understanding of the ecological role of terpenes produced by potato and other plants may result in highly specific applications and uses for such metabolites.

This further highlights the importance and role of allelopathy. The terpenes investigated, as plant natural products, are clearly released by their respective plants to confer a selective advantage. In the case of the potato, the release of Solanoeclepin A is hypothesised to assist in the evolutionary success of the plant, possibly as a defence mechanism against other pathogens. This is due to the fact that if Solanoeclepin A was redundant as a metabolite, it would be evolutionarily selected against, as it is clearly a disadvantage for the plant to signal its presence to Potato Cyst Nematodes. Therefore, the production of Solanoeclepin A must provide a selective advantage to the potato, and this must outweigh the negative effects of this. Understanding the biosynthetic pathway may shed light on the reason for the production of this compound, and could lead to the development of a solution against PCN. Understanding the biosynthesis may allow for potato varieties, which do not release Solanoeclepin A, to be bred; these varieties would therefore not stimulate nematodes to hatch. Biosynthesis understanding will also be crucial if genetic modification is to be used as a solution. The development of the CRISPR/Cas9 method of genome editing may be a useful tool for removing Solanoeclepin A biosynthesis from the potato.

*S*-Carvone is a monoterpene produced by the caraway plant. Such compounds are likely to be released in the wild as a form of allelopathy to prevent other plants in the vicinity from growing. This is demonstrated, as the monoterpene under investigation prevents the potato from sprouting. Understanding the ecological role of this monoterpene in the plant it is produced by,

and how this affects other plants in the vicinity, may result in a more potent sprouting inhibitor for the potato industry when compared to the conventional treatments. Knowledge of the plant's ecology may be highly useful for finding a solution to the huge losses which result from potato sprouting in storage.

Whilst natural products, such as the monoterpene discussed, may provide useful compounds for industrial processes, it is also important to consider the needs of the industry, in which these compounds will be used. In this case, any chemicals applied must not affect the processing quality of the potatoes. Furthermore, potato sprouting is a biological process with an array of key regulatory systems. Studies looking at sprout development require a holistic approach, in which many factors are considered, including potato variety and parameters used for determining 'sprouting'. Research into alternative sprouting inhibitors should provide environmentally friendly, economical solutions to this problem.

This study provides a basis on which further studies can be carried out, in order to improve the security of the potato crop in years to come. This will be essential for mitigating the imminent global food security crisis, which threatens countries worldwide, particularly in the face of an ever-expanding global population.

# 6 Appendices

## 6.1 Potato OSC sequence alignment

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CLUSTAL 2.1 multiple sequence alignment

BAS1            ------------------------GATCCTAATTAATATCATTAAATTGTAAGCCTCGTGA 37
DAS             ------------------------------------------------TTTCAGCCTGCTAA 14
BAS2            ------------------------------------------------------------
CAS             ------------------------------------------------------------


BAS1            ATAT--AGAAGAATTTAAGGATGTGGAAGTTGAAGATTGCAAAAGGACAAGATGATCCAT 95
DAS             ACTTGAAGAAGAAATCAAGAATGTGGAAATTGACGATTGCTCAAGGGCAAGATG---CAT 71
BAS2            --------------------ATGTGGAAATTGAAGATTGCTGAAGGGCAAAAAGGGCCAT 40
CAS             --------------------ATGTGGAAGTTGAAGGTTGCTGAAGG---AGGTAGTCCAT 37
                                    ********.****.*.****: ****   *..:.   ***


BAS1            ACTTGTACAGTACAAATAACTATGTTGGACGTCAAACATGGGAGTTCGACCCAAATGCTG 155
DAS             ATTTATACAGCACAAATAACTACGTTGGACGACAAACGTGGGAGTTCGACCCAAATAGTG 131
BAS2            ATTTGTACAGCACAAACAACTATGTTGGACGACAAACGTGGGAGTTCGATCCAAATGCTG 100
CAS             GGCTCCGAACGTTAAACGGTCACATAGGGCGACAAGTATGGGAGTTTGATCCGAACCTCG 97
                .  *  ..*  : *** ..  * .*:**.**:***. .******** ** **.**    *


BAS1            GAATGAAAGAAGAACATGCCGAGATCGAAGAGGCCCGCCAACATTTTTGGAATAATCGTT 215
DAS             GAACGGCAGAAGAACTGGCCGAGATTGAAAAGGCCCGTCAACAATTTTGGAACAATCATT 191
BAS2            GAACGATAGAAGAACGGGCTGAGATAGAAGAGGCCCGCCAACAATTTTGGAATAATCGTT 160
CAS             GGTCTCCGAAAGACTTGGAAGAGATTGAGAAGTTTCGCGCAGAGTTTTACAAAAATCGTT 157
                *.:    ..****.   *. ***** **..**   **  .* * ****. ** ****.**


BAS1            ACAAAGTTAAGCCCAATAGTGATCTTCTTTGGAGAATGCAGTTTCTAAGAGAGAAGAATT 275
DAS             ATAAAATCAAGCCTAATAGCGATCTTCTTTGGAGAATGCAGTTCCTCAGAGAGAAGAATT 251
BAS2            ATAAAGTCAAGCCTAGTAGTGATCTTCTTTGGCGAATGCAGTTTCTTGGAGAGAAAAATT 220
CAS             TTGAGACTAAACACAGCTCTGATCTTCTTATGCGCTATCAGTTTTCAAAGGAGAACCCCG 217
                : .*..  **.*. *. :  *********: *.*.:: *****    ...***** ..


BAS1            TCAAACAGAGAATTGGAGCAGTAAAAGTAGAAGAAGGAAAAGAAATTAGCCATGAAATTG 335
DAS             TCAAACAGAGAATTGGAGCAGTAAAAGTAGAAGAAGGAGAAGAAATTAGCCATGAAATTG 311
BAS2            TCAAACAAATAATTCCAGCAGTAAAAGTTGAAGAAGATGAAGAAATTAGTCATGAAGTTG 280
CAS             TTGGTACAATTCTGCCCCGAGTTCAAGTTAAAGATATTGGAGATATAACAGAAGATAATG 277
                * ..:...* :.*   .  ***:.****:.****:. :..***:**:*   *:**:.:**


BAS1            CTACAATTGCATTGCGTAGAGCTGTCCATTTCTTCTCAGCTTTACAGGCTACTGATGGAC 395
DAS             CTACAATTGCATTGCGTAGAGCTGTCCATTTCTTCTCAGCTTTACAGGCTACTGATGGAC 371
BAS2            CTACAATTGCATTGCATAGAGCAGTTAATTTCTTCTCAGCTTTACAGGCTAGTGATGGTC 340
CAS             TAGCCACCACGTTGAGAAGAGCCATCAGTTTTTATTCTACTCTACAGGCTCATGATGGTC 337
                 :.*.*  .*.***..:***** .* ..*** *: **:.** ********. ******:*


BAS1            ATTGGCCTGCTGAAAATGCCGGACCTCTCTTCTTTCTTCCACCTCTCGTTATGTGTATGT 455
DAS             ATTGGCCTGCTGAAAGTGCTGGACCTCTCTTCTTTCTTCCCCCTCTGGTTATGTGTATGT 431
BAS2            ATTGGCCTGCTGAAAATGCTGGTCCTTTGTTTTTTCTTCCACCTTTGGTCATGTGTATGT 400
CAS             ATTGGGCAGCAGATTGTGGAGGACCAATGTTTCTATTGCCTGGTTTGGTTATTGCTTTAT 397
                ***** *:**:**::.**  **:**: * **  *: * **   * * ** **   *:*.*


BAS1            ATATCACTGGGCATCTTAATACTGTATTTCCAGCTGAACATCGGAAGGAAATTCTTCGGT 515
DAS             ATATCACTGGGCATCTTAATACCGTATTCCCAGCTGAACATCGGAAGGAAATTCTTCGAT 491
BAS2            ATATCACTGGGCATCTTAATACTGTATTCCCAGCTGAACATCGAAAGGAAATTCTTCGGT 460
CAS             CTGTTACTGGGGCACTGAATGCAGTGTTATCTGAAGAGCATAAGCGAGAGATATGTCGTT 457
                .*.* ****** .:** ***.* **.**  *:*.:**.***......**.**:  *** *


BAS1            ATATATACTGTCACCAGAATGAAGATGGTGGATGGGGTTTGCACATAGAAGGTCATAGTA 575
DAS             ATATATACTGTCACCAGAATGAAGATGGTGGATGGGGTTTGCACATAGAAGGTCACAGTA 551
BAS2            ATTTATATTGTCATCAGAACGAAGATGGTGGATGGGGTTTGCACATAGAAGGCCACAGTA 520
CAS             ATCTCTACAATCATCAGAACTGTGATGGTGGGTGGGGTTTGCATATTGAAAGCCATAGTA 517
                ** *.** :.*** *****  .:********.*********** **:***.* ** ****


BAS1            CTATGTTCTGTACAACTCTGAGTTACATATGCATGAGGATCCTTGGAGAAGGACCAGATG 635
DAS             CTATGTTCTGTACAGCTCTGAGTTACGTTTGCATGAGGATCCTTGGAGAAGGACCAGATG 611
BAS2            CTATGTTCTGTACAGCTCTGAGTTACATTTGCATGAGGATCCTTGGAGAAGGACCAGATG 580
CAS             CCATGTTTGGTTCAGTTCTGAGCTATGTTACTCTGAGGTTGCTTGGTGAAAAGGCTAATG 577
                * *****  **:**. ****** ** .*::  .*****:* *****:***... *:.***


BAS1            GTGGCAAAAACAATGCTTGTGCTAGAGCAAGGAAATGGATTCTTGATCATGGTAGTGTCA 695
DAS             GTGGCGTAAACAATGCTTGTGCTAGAGCAAGGAAATGGATTCTTGATCATGGTAGTGTCA 671
BAS2            GCGGTGTAAATAATGCGTGTGCTAGAGCAAGGAAATGGATTCTTGATCATGGTAGTGTCA 640
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CAS             GTGG---AGAAGGGGCAATGGAGAAGGGCCGCAAATGGATTTTGGACCATGGTACTGCCA 634
                * **   *.* .. ** :  *. *..* ..* ********* * ** ******* ** **

BAS1            CTGCAATTCCTTCATGGGGGAAAACTTGGCTCTCGATTCTTGGAGCTTTTGAGTGGTTAG 755
DAS             TCGCCATTCCTTCTTGGGGAAAAACATGGCTCTCGATTCTTGGAGCTTTCGAATGGATAG 731
BAS2            CCGCAATTCCTTCTTGGGGAAAAACATGGCTCTCGATTCTTGGAGTTTTTGAATGGATAG 700
CAS             CTGCAATAACCTCGTGGGGGAAAATGTGGCTCTCAGTGCTTGGACTATTTGATTGGTCTG 694
                  **.**:.* ** *****.****  ********..* ******  :** ** ***: :*

BAS1            GAACCAATCCAATCCCCCCTGAGTTTTGGATTCTTCCATCTTTTCTTCCTATGAATCCAG 815
DAS             GAACCAATCCAATGCCACCTGAGTTTTGGATTCTTCCATCTTTTCTTCCCGTGCATCCAG 791
BAS2            GAACCAATCCAATGCCACCTGAGTTTTGGATTCTTCCATCTTTTCTTCCCGTGCATCCAG 760
CAS             GGAACAATCCACTTCCTCCAGAGATGTGGCTTCTTCCTTATATCCTTCCATTTCATCCAG 754
                *.*.*******.* ** **:***:* ***.*******:*.*:* *****  * .******

BAS1            CAAAAATGTGGTGTTACTGTCGAATGGTTTACATGCCAATGTCTTATCTCTATGGGAAGA 875
DAS             CAAAAATGTGGTGTTACTGCCGAACGGTCTACATGCCAATGTCTTATCTCTATGGGAAGA 851
BAS2            CAAAAATGTGGTGTTACTGTCGAATGGTCTACATGCCGATGTCTTACCTCTATGGGAAGA 820
CAS             GAAGGATGTGGTGCCATTGTCGTATGGTTTATCTGCCAATGTCTTACTTATATGGTAAAA 814
                 **..********  * ** **:* *** ** .****.********  *.***** **.*

BAS1            GATTTATTGGTCCAATCACACCTCTCATTTTGCAATTGAGGGTTGAGTTATATAATCAAC 935
DAS             GATTTGTTGGTCCAATCACACCTCTCATTTTGCAATTGAGGGAAGAGCTGTATGATCAGC 911
BAS2            GATTTGTTGGTCCAATCACGCCTCTCATTTTGCAACTGAGGGAAGAGTTATATGATCAAC 880
CAS             GGTTTGTTGGACCGATCACACCAACAGTCTTATCTTTGAGAAAGGAGCTCTTCACAGTCC 874
                *.***.****:**.*****.**:. ..* **. .: ****..: *** * *: ..: : *

BAS1            CATACGATGAAATTAACTGGAAAAGAGTACGCCATTTATGTGCAAAGGAGGATCTCTATT 995
DAS             CATATGATGAAATTAACTGGAAAAAAGTGCGCCATGTATGTGCTAAGGAGGATCTTTATT 971
BAS2            CATACAATGAAATTAACTGGAAAAAAGTGCGCCATGTATGTGCAAAGGAGGATCTCTATT 940
CAS             CTTATCATAAAATAAACTGGAATAAAGCACGCAATGAATGTGCAAAGGAAGACCTCTACT 934
                *:**  **.****:********:*.** .***.** :******:*****.** ** ** *

BAS1            ACCCTCATCCATGGGTTCAAGATTTGATGTGGGACAGTCTCAACATATGCACCGAGCCTC 1055
DAS             ACCCGCATCCATTGGTTCAAGATTTGATGTGGGATAGTCTCTACATATGTACCGAGCCTC 1031
BAS2            ACCCTCATCCATTGGTTCAAGATTTGATGTGGGACAGTCTCTACATATGTACCGAGCCTC 1000
CAS             ATCCTCACCCTCTACTACAGGACATCCTTTGGACATCTCTTGACAAGCTCATCGAACCTT 994
                * ** ** **:  . *:**.** :* .* ***.. : ***  ***:.   * ***.***

BAS1            TATTGACTCGTTGGCCTTTCAACAAGCTGAGAAATAAAGCTCTTGAAGTTACCATGAAAC 1115
DAS             TATTGACTCGTTGGCCTTTCAACAAGCTGAGAAATAAAGCTCTTGAAGTTACCATGAAAC 1091
BAS2            TATTGACCCGTTGGCCTTTCAACAAGCTGAGAAACAAAGCTCTTGAAGTTACCATGAAAC 1060
CAS             TGTTTATGCATTGGCCTGGGAAAAAGTTGAGGGAAAAAGCTCTTAGCACAGTAATGGATC 1054
                *.** *  *.*******   **.*** ****..* *********.... :. .***.*:*

BAS1            ATATACATTATGAAGACGAGAATAGCCGATACATCACCATTGGATGTGTTAATAAAGTAT 1175
DAS             ACATACACTACGAAGACGAGAATAGTCGATACATCACCATGGGATGTGTGGAAAAAGTAT 1151
BAS2            ACATACACTATGAAGACGAGAATAGTCGATACATCACCATTGGATGTGTCGAAAAAGTAT 1120
CAS             ACATACATTATGAAGATGAAAATACTCGCTATATATGCTTAGGGCCCGTGAACAAGGTCT 1114
                * ***** ** ***** **.****  **.** **.: *:* **.   ** .* **.**.*

BAS1            TGTGTATGCTTGCTTGTTGGGTTGAGGATCCCAATGGCGATTATTTCAAAAAACATCTCG 1235
DAS             TGAGTATGCTTGCTTGTTGGGTTGAGGATCCCAATGGCGATCATTTCAAAAAACATCTTG 1211
BAS2            TGTGCATGCTTGCTTGTTGGGTCGAGGATCCTAACGGCGATTATTTCAAAAAACATCTTG 1180
CAS             TAAATATGCTTTGTTGCTGGGTTGAAGATTCTAGTTCGGAAGCTTTTAAGTTGCATCTTC 1174
                *.:. ******  *** ***** **.*** * *.    **: .*** **.::.*****

BAS1            CTAGGATCCCAGATTATTTATGGGTAGCTGAAGATGGAATGAAAATGCAGAGTTTCGGT- 1294
DAS             CTAGGATCCCAGATTATTTATGGGTAGCTGAAGATGGAATGAAAATGCAGGGTTGTGGT- 1270
BAS2            CTAGGATCCCTGATTATTTATGGGTAGCTGAAGATGGAATGAAAATGCAGAGTTTTGGT- 1239
CAS             CACGATTATATGATTATCTATGGATTGCTGAAGATGGAATGAAAATACAGGGATATAATG 1234
                *:.*.:*. .:****** *****.*:********************.***.*:*  ..*

BAS1            --AGTCAAGCATGGGATACTAGTTTTGCTATTCAAGCATTATTGGCCAGTGAGATGAATG 1352
DAS             --AGTCAATCATGGGATGCTAGTTTAGCTATTCAAGCACTATTGGCCAGTGAGATGAATG 1328
BAS2            --AGTCAAGAATGGGATACCGGTTTTGCTATTCAAGCACTATTGGCCAGTGAGATGAATG 1297
CAS             GAAGTCAATCATGGGATACTTCTTTTGCTATTCAAGCAATCATTTCAACAAACCTTGTTG 1294
                  ****** .*******.*   ***:************ *.:*  *.* :.* .* .:**

BAS1            ATGAG-ATATTAGATACTCTGAGAAAAGGACATGACTTCATAAAAACATCGCAGGTGAAG 1411
DAS             ATGAG-ATATCAGATACTCTTAGAAAGGGACATGACTTTATAAAACAATCTCAGGTGAAG 1387
BAS2            ATGAG-ATAGCAGATACTCTTAGGAAAGGACATGACTTTATAAAACAATCTCAGGTGACG 1356
CAS             AAGAATATGGTCCA-ACTTTGCGAAAAGCACACAAGTTCATGAAAAACTCACAGGTGTTA 1353
                *:**. **.  . * *** * .*.**.* *** .* ** **.***...** ******: .

BAS1            GACAATCCTTCTGGTGATTTTAAAGGGATGTATCGACATATCTCAAAAGGATCGTGGACT 1471
DAS             GACAATCCTTCTGGTGATTTTAAAGGTATGTATCGGCATATCTCAAAAGGATCATGGGCT 1447
BAS2            GACAATCCTTCTGGTGATTTCAAAGGGATGTATCGACATATCTCAAAAGGATCGTGGACT 1416
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CAS             GATGATTGCCCAGGCAATCTTGATTTCTGGTATCGGCATATTTCAAAAGGGGCTTGGCCT 1413
                ** .**    *:** .** * .*:   : ******.***** ********. * *** **

BAS1            TTTTCAGATCAAGATCATGGATGGCAAGTATCTGATTCCACTGCCGAAGCATTAAAGTGC 1531
DAS             TTTGCAGATCAAGATCATGGATGGCAAGTATCTGATTGCACTGCCGAAGCATTAACGTGC 1507
BAS2            TTTTCAGATCAAGATCATGGATGGCAAGTCTCTGATTGCACTGCTGAAGCATTAAAGTGC 1476
CAS             TTCTCTACTGCAGATCATGGTTGGCCTATTTCAGATTGTACTGCGGAGGGACTAAAAGCA 1473
                **  *:..* .*********:****.:.* **:****  ***** **.* * ***..  .

BAS1            TGCCTTTTCTTCTCTACAATGCCTTCAGAATTAGTTGGTGAGGCAATCGAACCTGCACGA 1591
DAS             TGCCTTCTCTTCTCTACAATGCCTCCTGAATTAGTTGGTGAGGCAATGGATCCAGTGCGA 1567
BAS2            TGCCTTCTGTTCTCTACAATGCCTCGTGAATTAGTCGGTCAGGCAATGGAACCAGGGCGA 1536
CAS             TGTCTTCTACTCTCTAAACTACCTGTGGAAATCGTTGGTGAACCATTGAAGGCAAACCGT 1533
                ** *** *  ******.*.*.***   ***:*.** *** *. **:* .*  *:.  **:

BAS1            CTGTATGACTCAGTGGATGTTATTCTTACTTTCCAGAGCAAAAATGGGGGTTTAGCAGCT 1651
DAS             CTGTATGACTCGGTGAATGTTATTCTTTCATTACAGAGCAAGAATGGGGGTTTAGCAGGA 1627
BAS2            CTGTATGACTCGGTGAATGTTATTCTTTCATTACAGAGCAAAAATGGCGGTTTAGCAGCA 1596
CAS             TTGTATGATGCTGTAAATGTTATGCTGTCATTACAGAATCCTGACGGTGGCATTGGGACA 1593
                 *******  * **..******* ** :*:**.****. .. .* ** ** :*:* .. :

BAS1            TGGGAGCCAGCAGGGGCCTCACAGTATTTGGAGTTGCTCAATCCTACTGAACTTTTTGCG 1711
DAS             TGGGAACCTGCAGGGGCCGCAGAGTATTTGGAGTTGCTCAATCCTACTGAATTTTTCGAG 1687
BAS2            TGGGAACCTGCAGGAGCCTCTGAGTATTTGGAGCTGCTCAATCCTACTGAATTTTTTGCG 1656
CAS             TATGAACTGTCAAGGTCGTATCCATGGTTGGAGATAATCAACCCTGCTGAGACTTTTGGT 1653
                *. **.*   **.*. *  .: ..*. ****** *..**** ***.****.  *** *

BAS1            GACATTGTCATTGAGCATGAGTATGTTGAGTGCACTGGCGCATCAATCCAAGCACTAGTT 1771
DAS             GATATTGTTATTGAGCATGAGCATGTTGAGTGCACTAGCTCGGCAATCCAAGCACTTGTT 1747
BAS2            GACATTGTCATTGAGCATGAGTATGTCGAATGCACTGCCTCATCAATTCAAGCACTTGTT 1716
CAS             GATATTGTTATTGATTATCCTTACGTAGAGTGTACCTCAGCTATAATTCAAGCTTTGGCA 1713
                ** ***** *****  ** .  * ** **.** **   . *   *** *****: * * :

BAS1            CTGTTTAAAAAGCTATACCCTGGACACCGGACCACAGAGATTGACAATTTCATTGATAAT 1831
DAS             CGTTTTAAGAAGATATACCCTGGACACCGAACTACGGAGGTTGACAATTTTATTAATAAT 1807
BAS2            CTGTTTAAGAAGCTGTACCCCGGACACAGAACCAAGGAGATTAACATATTCATTGATAAT 1776
CAS             GCATTTAAGAAATTATACCCTGGGTATCGGAAAGAAGATGTGGAGCGTTGTATTGAAAAA 1773
                   *****.**. *.***** **. * .*.*. ...** .* .* . :*  ***.*:**:

BAS1            GCTGTTAAATATCTTGATGATGTACAGAAGCCTGATGGTTCATGGTATGGTTCCTGGGGT 1891
DAS             GGTGTTAAATATATTGAAGATGTACAGAAGCCTGACGGTTCATGGTATGGTAACTGGGGT 1867
BAS2            GCTGTTAAATATCTTGAAGATGTACAAATGCCTGATGGTTCATGGTATGGTAACTGGGGT 1836
CAS             GGTGCCGCCTTCATTGAAAAGATACAAGAAGCAGATGGCTCCTGGTATGGATGTTGGGGA 1833
                * **  ...*: .****:.* .****..:. *:** ** **.********::  *****:

BAS1            GTGTGCTTTACATATGCTTCCTGGTTTGCTCTTGGAGGGCTTGCTGCA-GCAGGCAAGAG 1950
DAS             GTGTGCTTCATATATGCTTCCTGGTTTGCTCTTGGAGGGCTTGCTGCT-GTAGGCTTGTC 1926
BAS2            GTTTGCTTCACATATGGTTCCTGGTTTGCTCTTGGAGGGCTTGCTGCA-GCAGGCAAGTC 1895
CAS             GTTTGCTTTACGTATGGCACATGGTTCGGGGT-GAAGGGCCTGCTGGATGCTGGGAGGAA 1892
                ** ***** * .****  :*.***** *   * *.***** ***** : * :** : *:

BAS1            TTACAGCAACTCTGCGGCTGTTCGAAAAGGCGTTGAATTTCTGTTACTAAGACAAAGGTC 2010
DAS             GTACAGAAACTGTGCAGCTGTTCGTAAAGGCGTAGAATTTCTTCTAAGAACACAAAGGTC 1986
BAS2            CTACAACAACTGTGCAGCTGTTCGTAAAGGCGTTGAATTTCTGCTAAGAACACAAAGGTC 1955
CAS             CTTCAACAATTCTTATAACATCCGTAAGGCCTGTGATTTTCTGTTATCAAAACAAGTAGT 1952
                 *:**..** * * . .. .* **:**.* *  :**:*****  **  ** ****. .

BAS1            TGATGGTGGTTGGGGAGAAAGCTATCATTCTTGTCCTGACAAGGTATATAGAGAGCTTGA 2070
DAS             TGATGGTGGTTGGGGAGAAAGCTACCGTTCTTGTCCTGACAAGGTATATAGAGAGCTTGA 2046
BAS2            CGATGGTGGTTGGGGTGAAAGCTACCGTTCTTGCCCAGACAAGGTATATAGGGAACTCGA 2015
CAS             GTCTGGTGGATGGGGAGAGAGTTATCTGTCTTGTCAAAACAAGGTGTATACAAATCTTAA 2012
                  .******:*****:**.** ** *  ***** *.:.*******.**** ..* ** .*

BAS1            AACAGAACACTCAAATCTTGTACAAACTGCATGGGCATTGATGGGATTGATTCACTCTGG 2130
DAS             AACAGAACACTCGAATCTTGTACAAACTGCATGGGCATTGATGGGATTGATTCACTCTGG 2106
BAS2            AACAAATGACTCAAATCTTGTACAAACTGCATGGGCATTGATGGGATTGATTCACTCTGG 2075
CAS             GGGTAACAAATCTCACAGTGTTTGTACTGCATGGGCTATGCTAGCTCTTATTGAAGCTGG 2072
                .. :.*  *.** .* . ***: .:***********::**.*.* : * *** *. ****

BAS1            CCAGGTT------------------------------------------------------ 2137
DAS             CCAGGTT------------------------------------------------------ 2113
BAS2            CCAGGTT------------------------------------------------------ 2082
CAS             ACAGGGA------------------------------------------------------ 2079
                .**** :

BAS1            --------------GATAGAGATCCAAGACCCCTCCACTGTGCGGCTAGGCTTTTGATTA 2183
DAS             --------------GATAGAGATCCAAGGCCCCTCCACCGCGCAGCAAAGCTATTGATTA 2159
BAS2            --------------GATAGAGATCCAAGGCCCCTCCACCGCGCAGCAAAGCTGTTGATTA 2128
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CAS             --------------GAGAGGGATCCAACTCCATTACACCGTGCAGCAAAGGTATTGATAA 2125
                              ** **.*******  **. *.*** * **.**:*.* * *****:*

BAS1            ATTCTCAAATGGAAGATGGTGACTTCCCACAACAGGAAATAACTGGAGTTGTCATGAAGA 2243
DAS             ATTCTCAGATGGAAGATGGTGACTTCCCACAGCAGGAAATAATTGGTGTTTTCTTGAGGA 2219
BAS2            ATTCTCAGATGGAAGATGGTGACTTCCCACAGCAGGAAATTACTGGAGTTTTTATGAAGA 2188
CAS             ATTCTCAGCTTGAAAATGGAGATTTTCCTCAGCAGGAGATAAGCGGGGTGTTCAACAAGA 2185
                *******..* ***.****:** ** **:**.*****.**:*  ** **  * :: *.**

BAS1            ATTGCATGTTGCACTATGCAGCATATAAAAATATATTTCCATTGTGGGGTTTGGCTGAAT 2303
DAS             ATTGTATGATGCACTATGCATCATATAGAAATATATTTCCATTGTGGGGATTGGCAGAAT 2279
BAS2            ATTGCATGTTGCATTATGCAGCATACAGAAATATATATCCATTATGGGGTTTGGCAGAAT 2248
CAS             ATTGCATGATATCGTATTCTGCATATAGGAACATCTTCCCAATTTGGGCTCTAGGACAAT 2245
                **** ***:*. . *** *: **** *..** **.*: ***:* **** : *.* : ***

BAS1            ACTG--CAAAAATGTCCAAGTACCATTAGTACACAACTAAATATATATTATA-------- 2353
DAS             ACCG--CAGAAATGTCTTAGTACCATTAAAACACAACTAAATATAGAGAATGCAATTAGT 2337
BAS2            ATCG--CAAAAATGTCCTATTACCATTAGAAAACAACTAA-------------------- 2286
CAS             ATCAGTCTCAGCTACTTAACCCTCAATGAGTGACAGTTATCAGTGAACTATATTTCACAG 2305
                *  .  *: *..*.   :*  . **:*.. : ***. **:

BAS1            --TTATGT---------------------CTTAA-GAAATGA--ACAAT--GTAGTTTAT 2385
DAS             CCTTACGTGGATTCCACGCATAGCGGGAGCTTAGTGAATCGA--GTTGTCCGTTGTTGTT 2395
BAS2            ------------------------------------------------------------
CAS             CTTC-TGCATTTCGTTCTT-----GAGTTTCTAGTTGAACAA-----GCAATGA-ATACT 2353


BAS1            TGTTTCTTGTG-TTAATGTAAG-----TTATGTAACAG---------------------- 2417
DAS             TGTTTGTTGTG-TTAATGTAAG-----TGATGTAACAATGTATAATATCATTATAATTCT 2449
BAS2            ------------------------------------------------------------
CAS             TGTTACTTGTG-AAATTGAACA-CTTTTTATTTATCACAAACATGCCTTGTGGAGT---- 2407


BAS1            ------------------------------------------------------------
DAS             CTTCAGAAAGTGATCTGTACTTCTATTATTCTATATAT---------------------- 2487
BAS2            ------------------------------------------------------------
CAS             ------------------------------------------------------------


BAS1            ------------
DAS             ------------
BAS2            ------------
CAS             ------------
```

**Appendix 1. Potato OSC sequence alignment.**

Potato OSC sequences (BAS1, BAS2, CAS, DAS) aligned using ClustalW.

## 6.2 Alignment of CAS from SpudDB and NCBI

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CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        ------------------------------------------------------------


CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        ------------------------------------------------------------


CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        ------------------------------------------------------------


CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        ----------------------------------------------------------ATGT 4


CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        GGAAGTTGAAGGTTGCTGAAGGAGGTAGTCCATGGCTCCGAACGTTAAACGGTCACATAG 64


CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        GGCGACAAGTATGGGAGTTTGATCCGAACCTCGGGTCTCCGAAAGACTTGGAAGAGATTG 124


CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        AGAAGTTTCGCGCAGAGTTTTACAAAAATCGTTTTGAGACTAAACACAGCTCTGATCTTC 184


CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        TTATGCGCTATCAGTTTTCAAAGGAGAACCCCGTTGGTACAATTCTGCCCCGAGTTCAAG 244


CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        TTAAAGATATTGGAGATATAACAGAAGATAATGTAGCCACCACGTTGAGAAGAGCCATCA 304


CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        GTTTTTATTCTACTCTACAGGCTCATGATGGTCATTGGGCAGCAGATTGTGGAGGACCAA 364


CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        TGTTTCTATTGCCTGGTTTGGTTATTGCTTTATCTGTTACTGGGGCACTGAATGCAGTGT 424


CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        TATCTGAAGAGCATAAGCGAGAGATATGTCGTTATCTCTACAATCATCAGAACTGTGATG 484


CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        GTGGGTGGGGTTTGCATATTGAAAGCCATAGTACCATGTTTGGTTCAGTTCTGAGCTATG 544


CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        TTACTCTGAGGTTGCTTGGTGAAAAGGCTAATGGTGGAGAAGGGGCAATGGAGAAGGGCC 604


CAS-SPUDDB      ----------------------------CACT---------------------------- 4
CAS-NCBI        GCAAATGGATTTTGGACCATGGTACTGCCACTGCAATAACCTCGTGGGGGAAAATGTGGC 664
                                            ****

CAS-SPUDDB      ---------------TATTTTGTT---------------CACT----------------- 17
CAS-NCBI        TCTCAGTGCTTGGACTATTTGATTGGTCTGGGAACAATCCACTTCCTCCAGAGATGTGGC 724
                               *****  **               ****

CAS-SPUDDB      ------CTTGT-----------------AGGAAGGATGTGGTGCCATTGTCGTATGGTTT 54
CAS-NCBI        TTCTTCCTTATATCCTTCCATTTCATCCAGGAAGGATGTGGTGCCATTGTCGTATGGTTT 784
                      *** *                **********************************

CAS-SPUDDB      ATCTGCCAATGTCTTACTTATATGGTAAAAGGTTTGTTGGACCGATCACACCAACAGTCT 114
CAS-NCBI        ATCTGCCAATGTCTTACTTATATGGTAAAAGGTTTGTTGGACCGATCACACCAACAGTCT 844
                ************************************************************

CAS-SPUDDB      TATCTTTGAGAAAGGAGCTCTTCACAGTCCCTTATCATAAAATAAACTGGAATAAAGCAC 174
CAS-NCBI        TATCTTTGAGAAAGGAGCTCTTCACAGTCCCTTATCATAAAATAAACTGGAATAAAGCAC 904
                ************************************************************
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CAS-SPUDDB      GCAATGAATGTGCAAAGGAAGACCTCTACTATCCTCACCCTCTACTACAGGACATCCTTT 234
CAS-NCBI        GCAATGAATGTGCAAAGGAAGACCTCTACTATCCTCACCCTCTACTACAGGACATCCTTT 964
                ************************************************************

CAS-SPUDDB      GGACATCTCTTGACAAGCTCATCGAACCTTTGTTTATGCATTGGCCTGGGAAAAAGTTGA 294
CAS-NCBI        GGACATCTCTTGACAAGCTCATCGAACCTTTGTTTATGCATTGGCCTGGGAAAAAGTTGA 1024
                ************************************************************

CAS-SPUDDB      GGGAAAAAGCTCTTAGCACAGTAATGGATCACATACATTATGAAGATGAAAATACTCGCT 354
CAS-NCBI        GGGAAAAAGCTCTTAGCACAGTAATGGATCACATACATTATGAAGATGAAAATACTCGCT 1084
                ************************************************************

CAS-SPUDDB      ATATATGCTTAGGGCCCGTGAACAAGGTCTTAAATATGCTTTGTTGCTGGGTTGAAGATT 414
CAS-NCBI        ATATATGCTTAGGGCCCGTGAACAAGGTCTTAAATATGCTTTGTTGCTGGGTTGAAGATT 1144
                ************************************************************

CAS-SPUDDB      CTAGTTCGGAAGCTTTTAAGTTGCATCTTCCACGATTATATGATTATCTATGGATTGCTG 474
CAS-NCBI        CTAGTTCGGAAGCTTTTAAGTTGCATCTTCCACGATTATATGATTATCTATGGATTGCTG 1204
                ************************************************************

CAS-SPUDDB      AAGATGGAATGAAAATACAGGGATATAATGGAAGTCAATCATGGGATACTTCTTTTGCTA 534
CAS-NCBI        AAGATGGAATGAAAATACAGGGATATAATGGAAGTCAATCATGGGATACTTCTTTTGCTA 1264
                ************************************************************

CAS-SPUDDB      TTCAAGCAATCATTTCAACAAACCTTGTTGAAGAATATGGTCCAACTTTGCGAAAAGCAC 594
CAS-NCBI        TTCAAGCAATCATTTCAACAAACCTTGTTGAAGAATATGGTCCAACTTTGCGAAAAGCAC 1324
                ************************************************************

CAS-SPUDDB      ACAAGTTCATGAAAAACTCACAGGTGTTAGATGATTGCCCAGGCAATCTTGATTTCTGGT 654
CAS-NCBI        ACAAGTTCATGAAAAACTCACAGGTGTTAGATGATTGCCCAGGCAATCTTGATTTCTGGT 1384
                ************************************************************

CAS-SPUDDB      ATCGGCATATTTCAAAAGGGGCTTGGCCTTTCTCTACTGCAGATCATGGTTGGCCTATTT 714
CAS-NCBI        ATCGGCATATTTCAAAAGGGGCTTGGCCTTTCTCTACTGCAGATCATGGTTGGCCTATTT 1444
                ************************************************************

CAS-SPUDDB      CAGATTGTACTGCGGAGGGACTAAAAGCATGTCTTCTACTCTCTAAACTACCTGTGGAAA 774
CAS-NCBI        CAGATTGTACTGCGGAGGGACTAAAAGCATGTCTTCTACTCTCTAAACTACCTGTGGAAA 1504
                ************************************************************

CAS-SPUDDB      TCGTTGGTGAACCATTGAAGGCAAACCGTTTGTATGATGCTGTAAATGTTATGCTGTCAT 834
CAS-NCBI        TCGTTGGTGAACCATTGAAGGCAAACCGTTTGTATGATGCTGTAAATGTTATGCTGTCAT 1564
                ************************************************************

CAS-SPUDDB      TACAGAATCCTGACGGTGGCATTGGGACATATGAACTGTCAAGGTCGTATCCATGGTTGG 894
CAS-NCBI        TACAGAATCCTGACGGTGGCATTGGGACATATGAACTGTCAAGGTCGTATCCATGGTTGG 1624
                ************************************************************

CAS-SPUDDB      AGATAATCAACCCTGCTGAGACTTTTGGTGATATTGTTATTGATTATCCTTACGTAGAGT 954
CAS-NCBI        AGATAATCAACCCTGCTGAGACTTTTGGTGATATTGTTATTGATTATCCTTACGTAGAGT 1684
                ************************************************************

CAS-SPUDDB      GTACCTCAGCTATAATTCAAGCTTTGGCAGCATTTAAGAAATTATACCCTGGGTATCGGA 1014
CAS-NCBI        GTACCTCAGCTATAATTCAAGCTTTGGCAGCATTTAAGAAATTATACCCTGGGTATCGGA 1744
                ************************************************************

CAS-SPUDDB      AAGAAGATGTGGAGCGTTGTATTGAAAAAGGTGCCGCCTTCATTGAAAAGATACAAGAAG 1074
CAS-NCBI        AAGAAGATGTGGAGCGTTGTATTGAAAAAGGTGCCGCCTTCATTGAAAAGATACAAGAAG 1804
                ************************************************************

CAS-SPUDDB      CAGATGGCTCCTGGTATGGATGTTGGGGAGTTTGCTTTACGTATGGCACATGGTTCGGGG 1134
CAS-NCBI        CAGATGGCTCCTGGTATGGATGTTGGGGAGTTTGCTTTACGTATGGCACATGGTTCGGGG 1864
                ************************************************************

CAS-SPUDDB      TGAAGGGCCTGCTGGATGCTGGGAGGAACTTCAACAATTCTTATAACATCCGTAAGGCCT 1194
CAS-NCBI        TGAAGGGCCTGCTGGATGCTGGGAGGAACTTCAACAATTCTTATAACATCCGTAAGGCCT 1924
                ************************************************************

CAS-SPUDDB      GTGATTTTCTGTTATCAAAACAAGTAGTGTCTGGTGGATGGGGAGAGAGTTATCTGTCTT 1254
CAS-NCBI        GTGATTTTCTGTTATCAAAACAAGTAGTGTCTGGTGGATGGGGAGAGAGTTATCTGTCTT 1984
                ************************************************************

CAS-SPUDDB      GTCAAAACAAGGTGTATACAAATCTTAAGGGTAACAAATCTCACAGTGTTTGTACTGCAT 1314
CAS-NCBI        GTCAAAACAAGGTGTATACAAATCTTAAGGGTAACAAATCTCACAGTGTTTGTACTGCAT 2044
                ************************************************************

CAS-SPUDDB      GGGCTATGCTAGCTCTTATTGAAGCTGGACAGGGAGAGAGGGATCCAACTCCATTACACC 1374
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CAS-NCBI        GGGCTATGCTAGCTCTTATTGAAGCTGGACAGGGAGAGAGGGATCCAACTCCATTACACC 2104
                ************************************************************

CAS-SPUDDB      GTGCAGCAAAGGTATTGATAAATTCTCAGCTTGAAAATGGAGATTTTCCTCAGCAGGAGA 1434
CAS-NCBI        GTGCAGCAAAGGTATTGATAAATTCTCAGCTTGAAAATGGAGATTTTCCTCAGCAGGAGA 2164
                ************************************************************

CAS-SPUDDB      TAAGCGGGGTGTTCAACAAGAATTGCATGATATCGTATTCTGCATATAGGAACATCTTCC 1494
CAS-NCBI        TAAGCGGGGTGTTCAACAAGAATTGCATGATATCGTATTCTGCATATAGGAACATCTTCC 2224
                ************************************************************

CAS-SPUDDB      CAATTTGGGCTCTAGGACAATATCAGTCTCAGCTACTTAACCCTCAATGAGTGACAGTTA 1554
CAS-NCBI        CAATTTGGGCTCTAGGACAATATCAGTCTCAGCTACTTAACCCTCAATGAGTGACAGTTA 2284
                ************************************************************

CAS-SPUDDB      TCAGTGAACTATATTTCACAGCTTC-TGCATTTCGTTCTTGAGTTTCTAGTTGAACAA-- 1611
CAS-NCBI        TCAGTGAACTATATTTCACAGCTTC-TGCATTTCGTTCTTGAGTTTCTAGTTGAACAA-- 2341
                ************************ ******************************

CAS-SPUDDB      GCAATGA-ATACTTGTTACTTGTG-AAATTGAACA-CTTTTTATTTATCAC--------- 1659
CAS-NCBI        GCAATGA-ATACTTGTTACTTGTG-AAATTGAACA-CTTTTTATTTATCACAAACATGCC 2398
                ******* *************** ********** ***************

CAS-SPUDDB      ------------------------------------------------------------
CAS-NCBI        TTGTGGAGT---------------------------------------------------- 2407


CAS-SPUDDB      ----------------
CAS-NCBI        ----------------
```

**Appendix 2. Alignment of putative potato CAS homologs from SpudDB and NCBI.**

## 6.3 Potato Council Fry Colour Chart


**Appendix 3. Potato Council Fry Colour Chart.**

Processed potatoes must be in categories 1-3 to be accepted by manufacturer (Kettle Chips Ltd.)

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