Sustainable Arable LINK Programme

Final Project Report

Project title:

Enhanced prediction of Susceptibility to Mechanical Damage in Harvested and Stored Potato Tubers

Sustainable Arable number: CSA6320 / LK0931

Start Date: March 2003

End Date: March 2006 (several items of the project continued after this date)

Partners: University of Durham
ADAS Consulting Ltd
Greenvale AP Ltd
McCain Foods (GB) Ltd
Branston Potatoes Ltd
Tesco plc
Martin Lishman Ltd
MBM Produce Ltd
British Potato Council

Project Manager: Dr Ron Croy

Report Date: December 2007

List of consortium personnel during LINK programme

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Huw Phillips	LINK PMC Monitor

Further details and contact addresses of the LINK consortium personnel are available in Appendix 1 supplied on the CD accompanying this report. These represent the personnel who have been associated with the programme either for the whole or for only parts of its duration. Other people may have been involved as representatives or stand-ins and apologies to any who have been missed off the list.

1.3 - List of figures

1.4 List of abbreviations

page

EPG – endopolygalacturonase 6
PME – pectin methylesterase
SO – superoxide radicals, superoxide anions
XTT - 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide
PMA - phorbol 12-myristate-13-acetate
AOS – active oxygen species
SOD – superoxide dismutase
PPO – polyphenol oxidase,
ECM – extracellular matrix
MTT - (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide)
NBT - nitroblue tetrazolium chloride
WST – Water-soluble tetrazolium salt
SDS – Sodium dodecyl sulphate

Section 2: Summary for Growers

page

Section 3: Experimental Sections, Results and Conclusions

page

Section 2: Summary for Growers

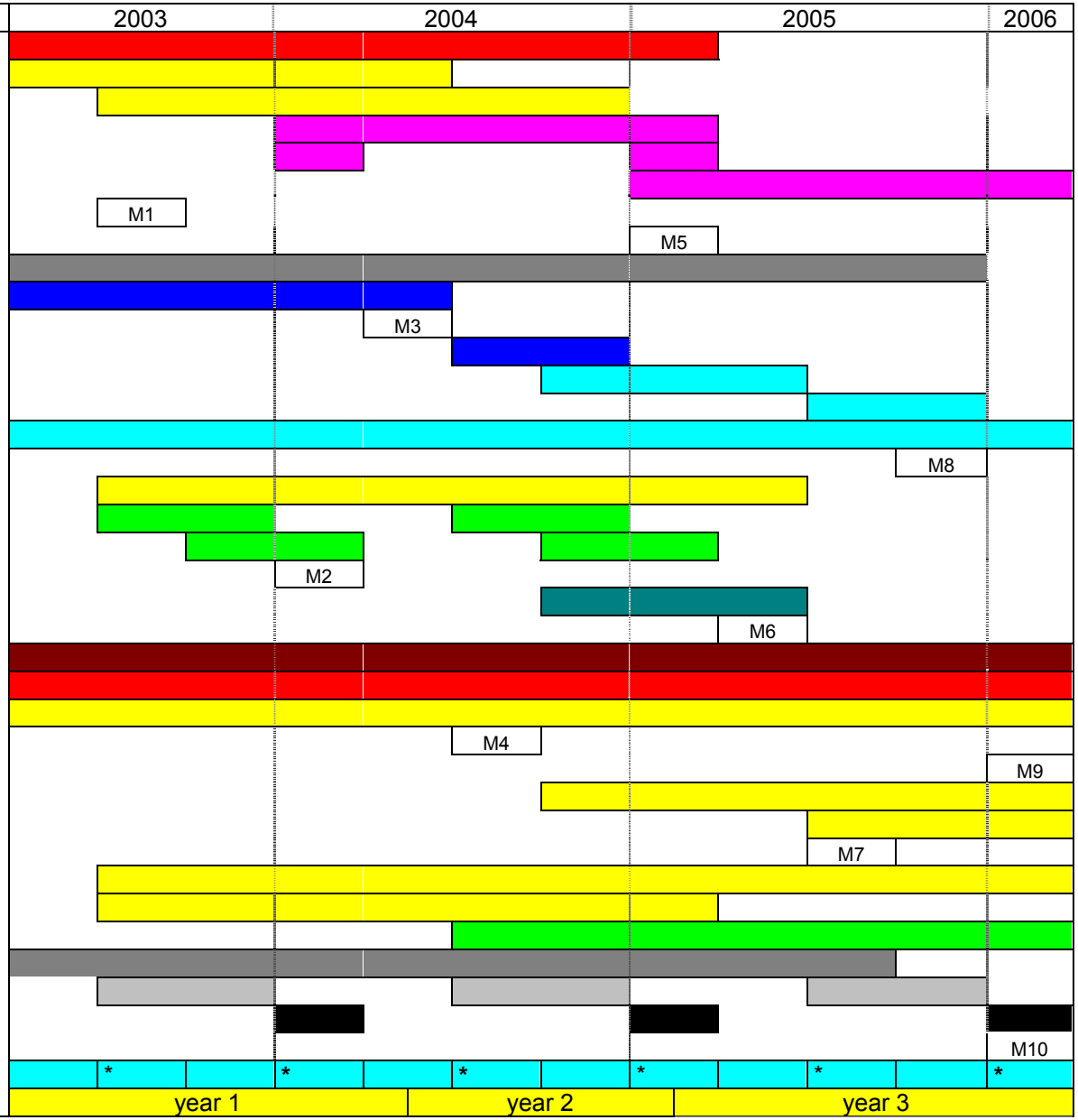
2.1 Project aims, objectives, tasks and milestones

The main aim of this project was to develop a diagnostic kit suitable for the field detection of susceptibility of potato tubers to mechanical damage in the form of blackspot bruising. At the start of this jointly funded project it had been established biochemically that the cells in mechanically damaged tuber tissue would generate a burst of highly reactive molecules known as free radicals. Free radicals are produced in plant tissues whenever they are exposed to stresses such as disease, adverse environmental conditions or physical damage. The previous work had established that the amount of free radicals generated by potato tuber tissues in response to a standard mechanical impact was proportional to the susceptibility of the tuber to the degree of blackspot bruising. Essentially the more susceptible tubers are to blackspot bruising the higher the level of free radicals generated by their tissues in response to an impact. The value of this observation is the opportunity to be able to diagnose bruise susceptibility in crops taken from the field or from potato stores before damage occurs. The results of such a test would allow informed decisions to be made about the subsequent handling and fate of susceptible crops. The aim of this project was to develop and test a kit by modifying the laboratory-based procedure and associated equipment for use by potato agronomists and growers in the field, packhouse or store. The objectives were to develop and optimize the components of a detection system in order for this to be a reliable means for detecting bruise susceptibility. The resultant diagnostic kit comprises of items of hardware to initiate free radical generation in a sample tuber, a device to excise a precise tissue sample, the detection chemistry for the sensitive detection of elevated levels of free radicals and a means to measure the intensity of the assay colour. Further objectives were to undertake fundamental studies on the biochemistry and molecular genetic studies of the response system in order to better understand the molecular bases of bruise susceptibility and resistance to mechanical damage. The value of these studies lies in the possibility of establishing further correlations between susceptibility or resistance and factors which might be more conveniently measured. This could facilitate the development of better means for the early detection of bruise susceptibility and to help us understand the basis of the correlation between bruise susceptibility and mechanical strength of tuber cells. The ultimate aspiration of these approaches would be to be able to predict a predisposition towards mechanical susceptibility and blackspot bruising early in the crop development and to adjust the crop treatment to rectify this problem.

The framework of the project is summarized in the Gantt chart shown below which lists the objectives and tasks and the milestones for measuring the progress of the project during its three years duration. In practice the fieldwork evaluations and some of the labwork extended for at least a further twelve – eighteen months beyond the final project completion date.

2.1a Gantt chart of objectives, tasks and milestones

Tasks	Description
Objective 1	**Optimising detection systems**
Task 1	New chemistries
Task 2	Optimising assays
Task 3	Establishing final assay procedure
Task 4	Risk assessment + evaluation
Task 5	Assembly and testing of prototype kits
Milestone 1	**preliminary kit format established**
Milestone 5	**evaluation of final assay**
Objective 2	**Hardware development**
Task 6	Design and build prototype 2
Milestone 3	**Production of prototype 2**
Task 7	Industrial evaluation of prototype 2
Task 8	Re-design and build final prototype
Task 9	Evaluation of final prototype
Task 10	Marketing + publicity + dissemination
Milestone 8	**Completion of hardware development**
Objective 3	**Field sampling methods**
Task 11	plant variation
Task 12	Industry trials and materials
Milestone 2	**Sampling strategy established**
Task 13	ADAS trials field variation / data handling
Milestone 6	**Finalised sample sizes for test**
Objective 4	**Commercial exploitation**
Task 14	define and assess market needs
Task 15	development of business plan
Milestone 4	**interim business plan**
Milestone 9	**full business plan for commercialisation**
Task 16	assess performance of prototype relative to market needs
Task 17	design for manufacture
Milestone 7	**review and secure IPR**
Objective 5	**Biochemistry of responses**
Task 18	Pectin and cell signals
Task 19	cell wall structure and composition
Objective 6	**Molecular genetics**
Task 20	potato samples selected extracted
Task 21	microarray preparation and assays
Milestone 10	**Publications**
meetings	**consortium 6 monthly meetings**



2.1b Project milestones taken from Gantt chart

Milestones from the original application

	description	target date
Milestone 1	preliminary kit format (hardware + chemistry) established	September 2003
Milestone 2	sampling strategy established	March 2004
Milestone 3	production of prototype 2	June 2004
Milestone 4	interim business plan	September 2004
Milestone 5	evaluation of final assay	March 2005
Milestone 6	finalised sample sizes for test	June 2005
Milestone 7	review and secure IPR	September 2005
Milestone 8	completion of hardware development	December 2005
Milestone 9	full business plan for commercialisation	March 2006
Milestone 10	publications	March 2006

2.2 Work undertaken and key findings

The work undertaken in this project broadly followed the objectives and tasks listed in the Gantt chart of the project schedule. As such the report discusses the results and findings from the various items of work under the headings of the objectives and tasks listed. The following is a brief summary of the work undertaken and the key findings from each.

1) The design, construction and testing of an impacting device (*impacter*) required to deliver a sufficient and reproducible force to initiate free radical generation in sample tubers. The project originally proposed the production of only a single prototype impacter based on the original model constructed in the previous Defra-BPC project. In practice a total of four pre-production impacters (Mks 2-5) were constructed during the course of the project incorporating improvements and modifications suggested by the results of laboratory experiments and by the field trials conducted by the partners. The criteria guiding the design of the impacters included the ease of use, stability in correctly locating the end of the barrel, efficiency in marking the impact site, reproducibility of impact delivery, ease of manufacture, portability. These criteria are discussed in more detail in **Part 2.2 – Impacter design** in the experimental **Section 3**. All impacter prototypes underwent extensive testing both in the laboratory as well as 'in the field' in the hands of the prospective end-users. The final designs proved to be reliable and capable of delivering a reproducible impact suitable for instigating radical generation. It was evident from the field evaluation that there could be a problem with any of the designs if a thorough cleaning regime is not implemented to keep the barrel free from soil particles and tuber material - this could not be alleviated by any design considerations. It became apparent during the field trials with the impacters that this device could also have great potential for use as a bruising tool, providing a convenient way of delivering an impact resulting in bruising at a precise position and which could be scored directly by a visual examination similar to the method used in this study. The impacter could substitute for less convenient equipment currently used for direct bruise assessment (bruise barrel, pendulum, falling bolt).

Strategies later in the project were directed towards the commercial manufacture of impacters for this purpose. (**See Part 4 – Commercialisation)**

2) The design, construction and testing of a sampling device (*corer*) suitable for the convenient excision of a precise and reproducible size of tissue sample from multiple potato tubers for testing for enhanced radical generation in the chemical assay. Again it was anticipated that a single prototype device would be designed and constructed based on the corer model completed in the previous programme. However a total of three additional pre-production coring devices were designed (Mks 2-4) and constructed, again incorporating the improvements suggested by 'in use' testing in the field and laboratory. Criteria used to guide the design included the efficiency of cutting the tissue cores, ease of use, long-term use and functionality. The final designs proved to be both efficient and easy to use and much of the fundamental work conducted later in the project was done using these corers. As with the impacters there could be a problem with any of these designs if a thorough cleaning regime is not carried out by the end users – this could not be avoided by any design considerations. Although a commercialization strategy to market the corers separately was not under consideration there could be an additional market for these devices as a convenient way of sampling fruit and vegetables for quality control tests or for scientific investigation.
3) Development and optimisation of the chemistry for detection of free radicals. The basis of the detection system was the reaction between a tetrazolium dye and superoxide free radicals to produce a coloured formazan product. The project work involved the selection of the best dye on the basis of several criteria – solubility, colour, intensity of colour change, stability of product, long term stability of dye (shelf life). This part of the work not only dealt with the detailed chemistry but also the format of the kit – the various consumables needed to carry out assays on a prescribed number of tuber samples. Risk assessments were carried out on the chemicals required for the assay to ensure that the selected reagents were not hazardous in use for the assay.
4) A further piece of hardware required for the kit was an electronic optical instrument (colorimeter) for measuring the intensity of colour generated by the radical assay. This was to substitute for the expensive research spectrophotometer instrument used in the lab-based assays. Selection of an appropriate colorimeter was based on criteria such as the cost, robustness of construction, clear readout, sample handling (tube size), accuracy of absorbance measurements compared to the spectrophotometer, portability (for field use), and the willingness of the manufacturer to modify the design to give a more appropriate display (reading). Two colorimeters that showed good accuracy were trialed over several growing seasons. The instrument selected had the advantages of robust construction, dedicated sample tubes and the manufacturer was willing to modify the readout (eg to bruise index) based on appropriate conversion information (**See Part 2.8 – Colorimeter evaluation**).
5) Trials of the diagnostic kit with field grown materials. The original and subsequent correlations between level of bruising and superoxide radical generation were established with precisely grown potato crops (ADAS), hand harvested and tested

in the laboratory. To fulfill the objectives of the project the kit had to be tested under field conditions and used in the manner for which it was developed. To achieve this kits were produced to allow the consortium partners to test them on selected field grown crops. Tests were conducted on tuber samples in the field, warehouse and from store. Difficulties were experienced throughout the field trials in trying to establish the tight correlation observed in the laboratory between superoxide radical generation and bruise susceptibility. No reliable predictions were possible using the kit and comparing the results to the conventional bruise evaluation methods. There was no clear reason for this failure but this problem is discussed in **Part 8 – A Commentary,** in the experimental **Section 3**.

6) During the project period and subsequently a large number of fundamental studies were also undertaken. Preliminary studies to investigate cell and tissue mechanical strength directly showed some promising correlations which might indicate the basis for the bruise susceptibility correlations and mechanical susceptibility. A major study was conducted into the biochemistry of the cellular responses following mechanical impact. These provided some insights into the nature of the signaling and enzymic reactions taking place during the biphasic response in susceptible tuber tissues **Part 5 - Studies on the biochemical and physiological responses of tuber cells to impact**.
7) Transcript profiling was also carried out on a range of tuber samples of known bruise susceptibilities. Preliminary analyses of these results have shown a number of genes to be differentially regulated between bruise susceptible and bruise resistant tubers. This aspect of the work will be continued to complete the large-scale analysis of the differential expression of tuber genes. See **Part 6 - Molecular genetics – transcript profiling**.

2.3 Conclusions and important points relevant to producers

This extensive programme has provided new hardware and a large amount of data much of which is immediately relevant to the potato industry or could be in the future if some of the recommendations are followed. Clearly the work on the development of the Blackspot Detect kit components has been a highly successful collaboration between the prospective end-users and the scientists and engineers involved in the design and optimization of the hardware and chemistry. The development of the hardware components in the form of impacter, corer and colorimeter has ultimately provided devices which are fit for purpose incorporating the features required by the potato companies for use 'in the field'. Although impacter and corer devices are integral components of the bruise diagnosis kit, independently they have added utility as test equipment for alternative bruise testing and for tissue sampling for quality control. Plans for the commercial manufacture and quality control of the impacter (Lishman Bruiser) are underway (See **Part 4 – Commercialisation**).

A major disappointment in the development of Blackspot Detect, the diagnostic kit, has been the failure to establish correlation between degree of bruising and level of SO generation using the established chemical assay in field tests. The exact reason for this is unclear although a commentary with possible explanations for the loss of correlation is provided in the experimental **Section 3** (**Part 8**). Much time has been spent on checking

the assay chemistry and stability of reagents but this does not seem to be at fault. A re-examination of the laboratory assay procedures closely compared to those executed in the field trials and by the same investigator might shed some light on the problem. The principle of the chemical test and the biological phenomenon of radical generation in response to mechanical impact are firmly established and further experimentation has revealed some of the nature of the signaling and response biochemistry (**Part 5**).

Prior to this project the industry used averaging techniques for estimating bruise damage of harvested crops (**hot** box to reveal naturally incurred bruise damage, **bruise barrel** to expose tuber samples to some measure of a standard mechanical impact). This is an adequate quality control for large-scale processing and ware crops but doesn't provide any information on the variability of bruising between tubers in the sample. This is important because the Blackspot Detect test uses much smaller samples to estimate the condition of a large crop ie. the crop from a whole field. With the development of the impacter the opportunity has arisen to perform more discriminating tests on individual tubers. This has revealed a significant variability in bruising in individual tubers as judged by the many scatter plots produced by the industrial partners. Different commercial companies use their own techniques for bruise damage assessment which make it difficult to compare results. It would be a useful step forward to produce a commercial impacter along with a bruising standard test to allow direct comparison of results from different growers.

Exploitation plans: At the point of the completion of this project in March 2006 and after two sets of field trials the results were not conclusive and there was still difficulty in establishing a correlation observed in the lab trials using field grown potato crops. As a result the Consortium agreed that more data and data analysis were needed from additional field trials and to provide improved sampling recommendations for use in the commercial bruise susceptibility test kit. Several of industrial members of the consortium including BPC were sufficiently convinced of the value of the kit concept to invest the additional finances needed to support these further field trials during the 2006 season (i.e. post LINK grant). Although outside of the LINK programme and financed separately some of the conclusions of the work by the industrial partners is described in this report.

2.4 Practical recommendations

1. Develop the impacter and corer as stand-alone commercial tools for the industry.
2. Re-investigate the correlation between radical generation and bruise susceptibility. This may require corroborating the original laboratory conditions but then it should be followed by applying the assay to the field test situation preferably by the same individual.
3. Examine the results of the detailed biochemical and physiological studies of tuber tissues to establish any correlation between measurable factors and bruise susceptibility / resistance.
4. Complete the transcriptomic analyses, data mining and associated bioinformatics – devise a model relating specific and relevant gene expression to bruise susceptibility / resistance.

Section 3: Experimental Sections, Results and Conclusions

In such a diverse and multifaceted project the descriptions and results from the many different activities have had to be reported in many separate sections. The plan of the report essentially follows the format requested by the funding agencies but is divided up into sub-sections corresponding to the different objectives and tasks as originally listed in the grant proposal. As appropriate some objectives may be dealt with in the same sub-section where the work reported is of similar nature. Some of the tasks associated with different objectives may not be reported in the order listed.

Part 1 - Studies relating to the optimization of the detection system and the relationship between superoxide production and bruise susceptibility.

Objective 1 – Optimising the detection systems

Task 1 - New chemistries
Task 2 - Optimising the assays
Task 3 - Establishing the final assay procedure
Task 4 - Risk assessment and evaluation
Task 5 - Assembly and testing of prototype kits

1.1 Introduction: The diagnostic kit (**Figure 1.1**) comprises of various items of hardware and a chemical assay system. The previous BPC and Defra funded projects led to the development of a chemical detection system designed to quantify the levels of superoxide radical generation by tuber cells in tissue explants. The basis of the chemistry has been reported in previous documents, published papers and the sponsor reports but it is described here briefly for the sake of completeness and to identify where changes to the chemistry or optimization has taken place. Tetrazolium dyes or salts are a series of redox indicator compounds which can undergo reduction to a formazan derivative producing a distinctive colour change. Tetrazolium compounds react readily with superoxide radicals to produce the coloured formazans however many of these formazans are insoluble in aqueous solution making measurement of product concentration difficult. Developments in tetrazolium chemistry have led to the production of a new generation of dyes known as water-soluble tetrazoliums (WST's) which produce soluble coloured formazan products making a colorimetric assay for quantifying superoxide generation by viable cells a possibility. XTT was one of the first WST's produced and was initially selected for this project because it was available commercially (**Sigma – Aldricht**) at reasonable cost. XTT is a yellow solid which dissolves in aqueous buffer to produce a pale-yellow almost colourless solution which reacts with superoxide to produce an orange formazan (**Figures 1.2 and 1.3**).

Figure 1.1 Early versions of the original "Blackspot Protect" kit. Components comprised impacter, corer, colorimeter and detection chemistry. The kit was renamed Blackspot Detect later in the project.

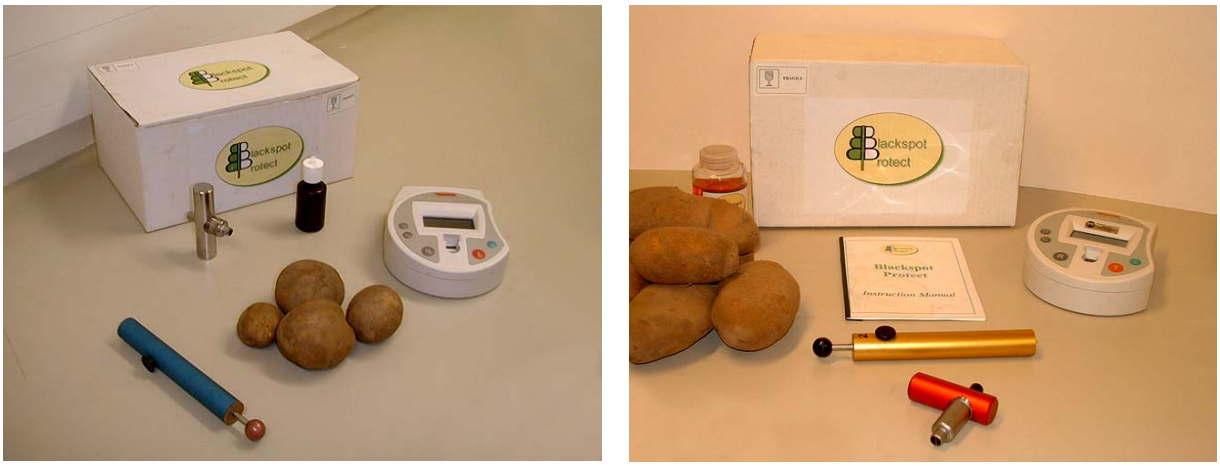
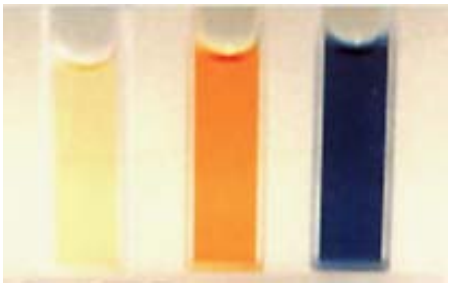
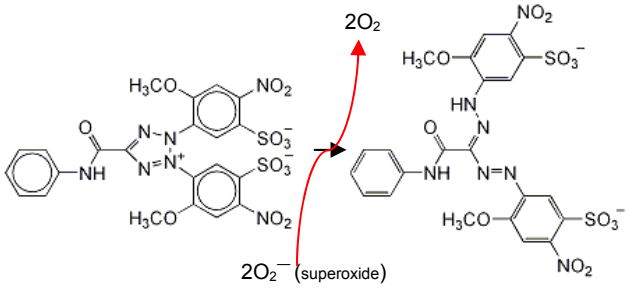


Figure 1.2 – XTT tetrazolium dye information





XTT = Sodium 3,3,-[(Phenylamino)carbonyl]-3,4-Tetrazolium-Bis(4-methoxy 6-nitro) benzenesulfonic acid hydrate)

XTT formazan @ pH7.0 formazan @ pH11.5

XTT formazan

molecular structures

Free radicals are extremely reactive and will react with any molecules (lipids, proteins, nucleic acids) in close proximity to their point of generation. **Superoxide** (O_2^-) is a reactive anion and a free radical of oxygen. It has an unpaired electron, is not particularly stable ($t^{½}$ <µsec), and spontaneously decomposes into peroxide and oxygen over time. It is biologically quite toxic and mechanisms to remove it are widespread in cells. In order to detect the superoxide radicals generated by the tuber cells it is necessary to immerse the whole tissue explant in the tetrazolium dye solution so that as soon as the radicals are generated outside of the cell the tetrazolium dye sequesters them before they can react with any other molecules. In this way the assay becomes quantitative since all radicals are reacted with XTT to produce the formazan product in a molar equivalent manner. Thus actual amounts of superoxide radicals generated can be calculated. The mechanism whereby superoxide radicals are generated in response to a mechanical impact is not fully understood but almost certainly involves activation of a plasma membrane NADPH oxidase (superoxide synthase) enzyme complex. The lag period of 1-2h between impact and the generation of superoxide as originally described by Johnson *et al.* (2003) supports this mechanism. Presumably the enzyme complex is activated in all the cells within the impact zone so that total radical generation is substantial. Once activated the enhanced generation of superoxide continues for up to two hours with maximal generation occurring at about 2h post impact, before declining followed by a second peak of radical generation at about 4h post impact in the characteristic biphasic (2 peak) response described by Johnson *et al*. (2003). Furthermore lab-scale experiments determined that the level of superoxide generated is directly correlated with the level of bruising following mechanical impact. Thus superoxide generation becomes diagnostic for tuber bruise susceptibility and mechanical damage. The underlying basis for the correlation has not been determined although several of the other approaches used in this LINK project have indicated possible mechanisms and ways to elucidate the correlation.

1.2 Experimental materials: The bulk of the experimental tuber materials were grown by ADAS mainly at their experimental field facilities at Gleadthorpe. Tubers from defined field plots were hand lifted, size sorted, bagged and transported for cold storage at ADAS, High Mowthorpe. Some additional tuber materials were provided by the consortium industrial partners and BPC, Sutton Bridge Experimental Station. Other materials are as specified in the relevant experimental sections.

1.3 Bruise assessments: Throughout the project it was standard practice to assess the bruising status of the experimental tuber materials held in storage or taken direct from the field. In order to obtain a valid assessment of blackspot bruise susceptibility within the test varieties a standardised bruise assessment test was established based on methods used in previous projects. 25 tubers per variety were mechanically impacted using a single impact from an impacter or falling bolt (see previous BPC and Defra reports). After complete bruise development (48 hours at 37°C) each tuber was bisected through the impact

zone and a measure of the width and depth of pigmented tissue was taken along with a visual assessment of the bruise pigment intensity compared with the surrounding tissue, using the following scale:

0 – No visible pigmented tissue at site of mechanical impact
1 – Low level of pigmented tissue (typically pink, red, red-brown, grey)
2 - Intermediate level of pigmented tissue (typically brown or brown-black)
3 - High level of pigmented tissue (typically blue-black or black)

The mean for bruise depth, width and pigment assessment was calculated and this was used for comparing blackspot bruise susceptibility between different varieties, or between different storage ages of tubers from the same variety. Bruise index was calculated by using equation 1.

Equation 1

$$\frac{\pi \times \frac{1}{2}(\text{bruise width})^2 \times \text{bruise depth} \times \text{bruise intensity}}{235.6}$$

This assumes a cylindrical shaped bruise zone and by dividing by 235.6 this compares the bruise index to a bruise with diameter 10mm, depth 10mm and bruise intensity 3 - theoretically an extensive and highly intense bruise which would not actually be observed in practice. This method of calculating a bruise index has several advantages over previously used methods – it minimises the subjective element as far as possible to only 4 visual categories of bruise pigment levels (=level of pigment synthesised); it factors in the size (volume) of the bruise zone as well as the bruise intensity, and furthermore it allows for easy comparison of bruise indices on a scale of 0 - 10. Quantitation of bruise pigmentation levels by measuring the grayscale intensity on a digitised image of the bruise zone was considered early in these projects but abandoned because this approach would be prohibitively labour intensive considering the numbers of tubers to be assessed.

1.4 The superoxide assay: The lab-based diagnostic scheme as established by the end of the previous project is illustrated in **figure 1.2** below. During the course of the project various changes to the procedure have been introduced either to address chemical or hardware problems or to accommodate the practicalities of the kit for use in the field. These changes are discussed in the relevant sections below. The fully detailed and amended protocol appears in **Appendix 2 - Blackspot Detect Instruction Manual 2005_2006**, accompanying this report but briefly the assay comprises of the following steps:

1. Sufficient assay solution is prepared – XTT tetrazolium dye solution in 50mM potassium phosphate buffer pH 6.8-7.2. This solution is made freshly and stored in the dark (foil wrapped) at room temperature until required.
2. Selected tubers are cleaned (optional) and a site selected for impacting near the stolon end of each test tuber.
3. The impacter is primed ready to deliver the impact and its barrel is positioned against the impact site.
4. Sufficient pressure is exerted so that the marking device (see later) locates the position of the impact and good contact is made between the impacter head and the tuber surface.
5. The impacter is fired. The point of impact is noted and marked clearly if necessary.
6. Impacted tubers are incubated in the dark for 2h or 4h at 37^{o}C.
7. After incubation a sample of tuber tissue (2 half cores) is removed from the impact zone.
8. One half core is washed x3 in distilled water and then immersed directly into the 200µl of the assay solution and incubated for 20 minutes at room temperature (20^{o}C)
9. The assay solution is passed through a membrane filter to remove any particulate materials (starch, cells, cell walls).
10. The clear orange formazan solution is read in a spectrophotometer or colorimeter set to a wavelength of 450-470nm.
11. The quantity of superoxide produced can be calculated from the molar extinction coefficient of XTT = 23600 M^{-1} cm^{-1}
12. (Optional) The pH of the assay solution containing the formazan product is adjusted to pH 11-12 by addition of a standard volume of sodium hydroxide solution and the solution read at a wavelength of 600nm (See later experimentation on this step in **Section 1.5**).

Figure 1.3 – The original bruise susceptibility detection method.
This procedure was established in the previous project and formed the basis of the Blackspot Detect test

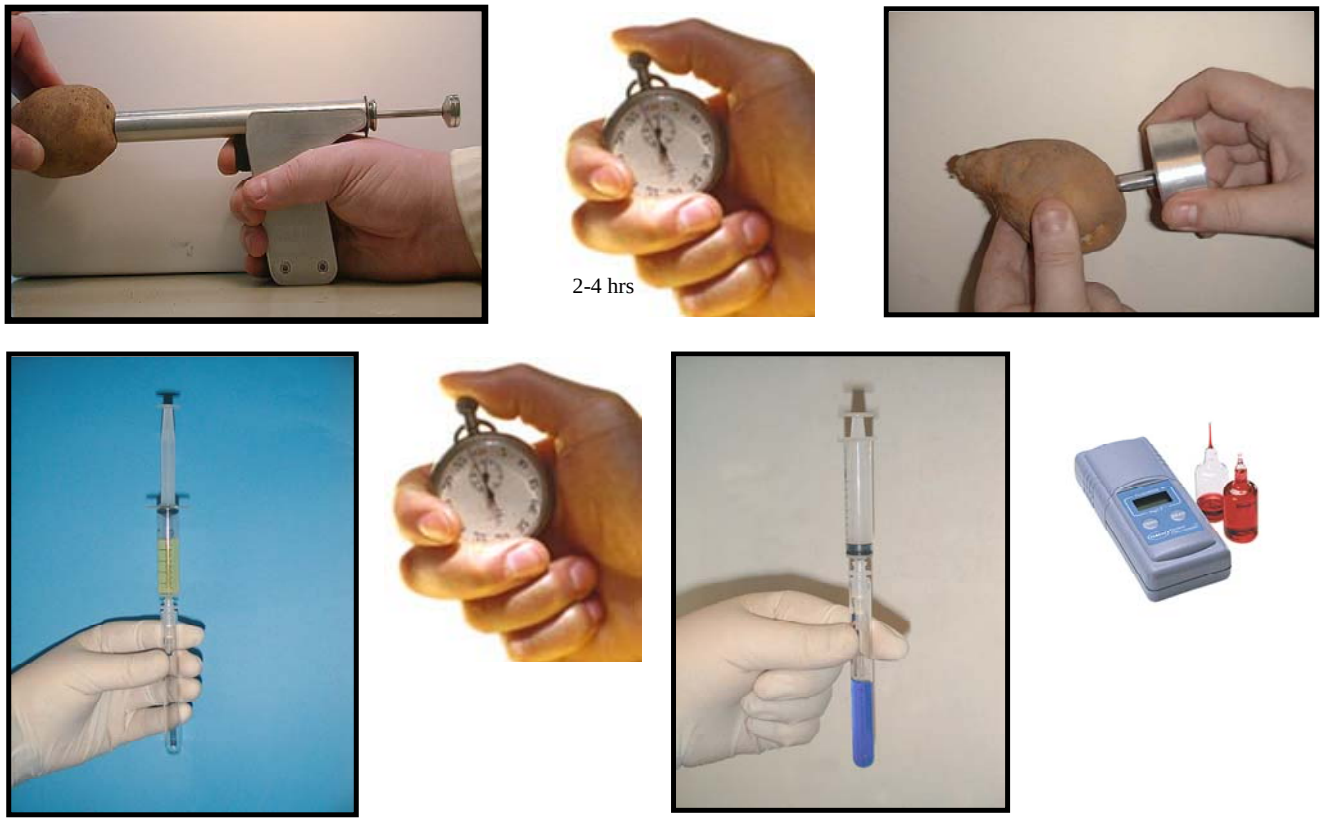
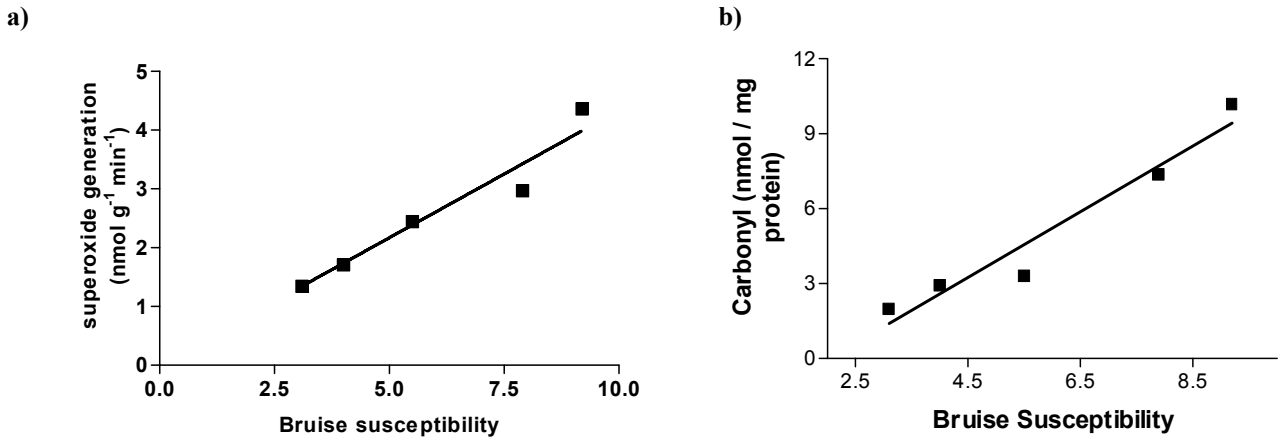


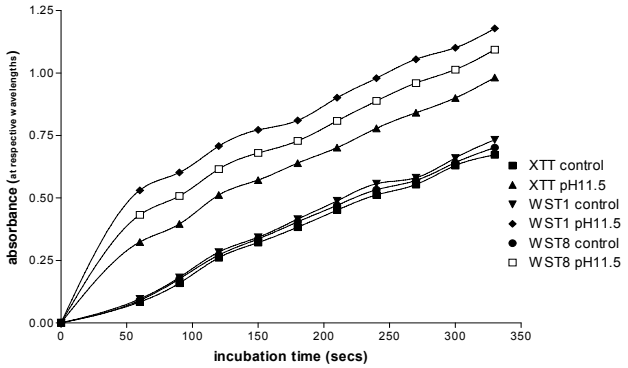
Figure 1.4 Correlation between radical generation and bruise susceptibility. The original correlation between a) superoxide generation and tuber bruising and b) level of radical-induced protein secondary carbonyls and tuber bruising as reported in Johnson *et al* (2003). The Pearson R^2 value of correlation 0.9448 in **Figure 1.4a)** indicates a very high level of correlation. Cultivars used to establish this correlation were Russet Burbank ; Saturna ; Cara ; King Edward and Maris Piper. Data are the mean of three replicates. The carbonyl assay provided corroboration of the direct measurement of radical generation.



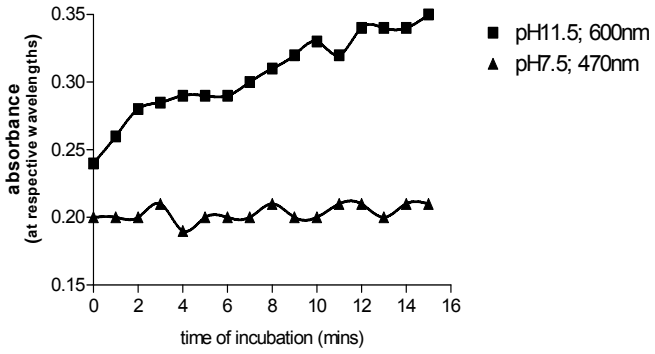
1.5 Alkalinisation of the pH of the formazan product solution: Tetrazolium dyes and the formazan derivatives are pH indicators – they change colour by altering the pH as well as by changing the redox status. From our preliminary experiments it was anticipated that we could employ this pH-induced colour change to enhance the colour yield of the product thereby increasing the differential between XTT and formazan absorbances (pale yellow > dark blue compared with pale yellow > pink / orange) making the overall assay more sensitive. This had the added advantage of potentially providing end-users with a simple visual assay of bruise susceptibility by using a reference colour chart (see later). Alkalinisation has been suggested by the tetrazolium dye suppliers for improving sensitivity in several tetrazolium-based assays. Initial investigations using an artificial superoxide chemical generator (see also **Part 1.8**) with XTT and new WST dyes (see **Part 1.6**) showed that there was indeed a significant enhancement in the relative absorbances. The results, plotted in **Figure 1.3a**), show the linear formazan production for each of the dyes XTT, WST1 and WST8 at the normal assay pH (pH7.5) and then the absorbances were measured immediately at the appropriate wavelengths, after adjusting the pH to 11.5 without an incubation period. However in subsequent more detailed experiments with XTT, the absorbance of the alkalinized solution was

Figure 1.5 Effect of alkalinisation of the formazan product in the superoxide assay

a) WST reaction with superoxide vs pH



b) Increasing absorbance of XTT formazan at pH11.5



followed for up to 20 minutes after addition of alkali (**Figure 1.3b**). It was immediately obvious that the absorbance was changing in a manner independent from the superoxide-induced change. Over a period of 15 minute incubation the absorbance increased by almost 50% over the starting value. Over the same time course the normal, control assay (pH7.5) showed no change in absorbance – in fact the formazan product at this pH is probably stable for up to an hour. The nature of this instability is unknown but is likely to be a problem with any of the available tetrazolium dyes since they all have very similar structures – alkaline-induced hydrolysis similar to the reductive radical cleavage to produce the formazan products is the most likely cause.

Conclusions: in view of the short-term instability of the absorbances of the pH adjusted formazan solutions, combined with the possibility of delays during field assays in measuring the absorbances of large numbers of assay solutions, this step might create more problems than would be worth for the gain in sensitivity. Changes in absorbance would add significantly to the variability in the assay results. Most likely the alternative tetrazolium dyes to be tested (see later) would behave in a similar way since these too are pH indicators.
Recommendations: Use of this step to be discontinued in all subsequent developments of the assay chemistry.

1.6 Alternative chemistries - the water soluble tetrazolium salts: In addition to XTT several new tetrazolium dyes became available at the start of this project. These were developed by a Japanese company (Dojindo) for use in applications to measure cell viability through respiratory activity. The benefit of these dyes, like XTT, is that when they undergo reductive cleavage the formazan product remains soluble in the aqueous assay solution rather than precipitating out of solution like earlier tetrazolium products such as MTT* and NBT**. WST is the generic term for these proprietary water soluble tetrazolium salts which are simply referred to as WST-1, WST-3, WST-4, WST-5 etc. A selection of these dyes were obtained from NBS Biologicals, Cambridgeshire. It was noted that the WST dyes were significantly more expensive than XTT. A quotation obtained early in the project indicated that the price per 100mg WST would be £195 (Nicolas Namur, NBS Biologicals) which is more than double the cost of an equivalent weight of XTT (actual prices would be somewhat less than this if purchased in bulk). Enquiries on bulk discounts on the supply of XTT by BioVectra Inc, Prince Edward Island, Canada failed to secure a significantly better pricing than Sigma on quantities less than about a 100g (worth several hundred thousand pounds). A variety of experiments were carried out with these dyes alongside XTT, to assess their utility and properties for use in the superoxide assay. Results shown in the preceding section (**Part 1.5**) indicated that WST-1 and WST-8 were suitable alternatives for XTT (but without the alkalinisation step) but showed no real advantage over it. Some of the dyes were only available as concentrated aqueous solutions which was undesirable for large scale kit production and could be a concern for storage and stability. Of key concern for the use of tetrazolium dyes for the kit preparation were the reports that the dye solutions and the solid dyes were unstable even during short term storage especially when exposed to the sunlight and fluorescent lighting. It was desirable to provide as long a 'shelf-life' as possible by selecting tetrazolium dyes which were significantly more stable as solids or in solution than XTT. This could avoid end-user problems later with failed or erroneous assays due to loss of activity of the radical detection areagent. To this end samples of WST dyes and XTT were prepared as 10ml assay strength solutions and aliquots of these and samples of the solid dyes were then held in storage at a range of temperatures – frozen at -20^{o}C; +4^{o}C and +20^{o}C. The remaining tetrazolium contents of the solutions and solid were estimated using spectrophotometry (absorbance at the respective wavelengths). The results were calculated as percentage remaining activity and are shown in **Table 1.1.** The stabilities of most of the WST dyes were comparable to XTT, in solution at -20^{o}C they retained most of their activity. This is the temperature for storage of tetrazolium dye solutions as recommended by the suppliers. The apparent high loss of activity of WST4 at 4^{o}C cannot be explained but XTT and the other WST dyes retained at least 90% of their activity in solution at this temperature indicating that storage for short periods in a domestic 'fridge without significant loss of activity is a possibility. All dye solutions were inactivated by storage at 20^{o}C although no time points of less than a month were tested and work reported elsewhere indicated that at least XTT solutions were stable overnight at room temperature. Surprisingly these results imply that up to 25% of the activity was lost by storage of the solid dyes at room temperature. This is at variance with the results obtained in a subsequent, more detailed study (See **Part 1.8**).

Table 1.1 – Stability of WST and XTT tetrazolium dyes. The tetrazolium dyes were stored at different temperatures in the dark. Figures are percentage remaining dye activity after storage for 1 month under the conditions indicated.

temperature		XTT	WST1	WST3	WST4	WST5
Solutions at	**- 20^{o}C**	100	99	100	100	100
	+4 ^{o}C	91	93	90	72	94
	+20 ^{o}C	0	0	0	0	0
Solid at	**+20 ^{o}C**	78	80	77	76	81

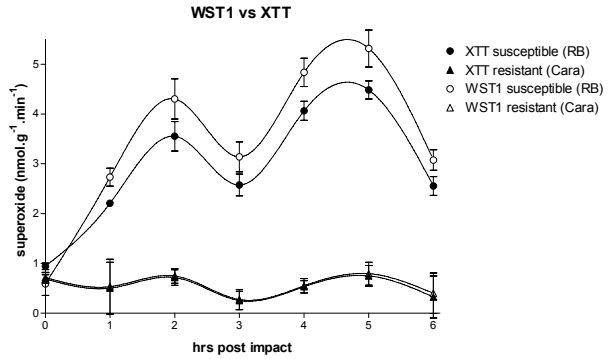
The stability results with the various dye solutions were encouraging in that solutions were sufficiently stable at 4^{o}C to provide the opportunity for making up bulk XTT or WST stock solutions and storing them in a refrigerator for use over a few days. -20^{o}C would be even better for longer storage periods but less convenient. Clearly medium-term storage of dye solutions at room temperature is not an option although satisfactory assays could be performed using XTT solutions stored in the dark for up to 24h (data not

shown). This property may be more relevant to the kit manufacturing process than for extending the usability of the kit reagent by end-users.

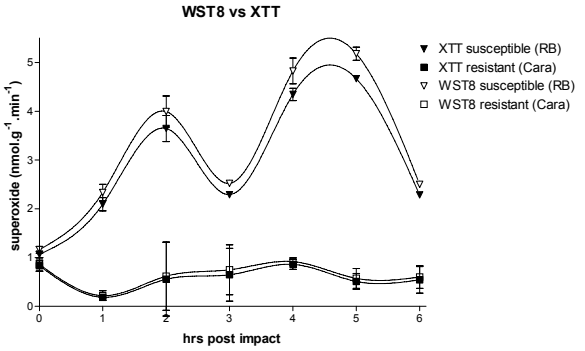
A longer term and more comprehensive storage study on various XTT samples prepared for kit use, was carried out after the end of the project and the results of this are presented in **Part 1.8**. Two of the WST dyes were also tested in the standard superoxide assays. Two tubers of resistant (*Cara*) and susceptible (*Russet Burbank*) varieties were tested in the time course experiment that produces a biphasic superoxide response as described previously (Johnson *et al.* 2003). This experimental system produces a wide range of superoxide generation (from zero up to the maximum for a susceptible tuber variety) so was ideal for comparing the dye performance at very low and very high superoxide levels. Consult the paper for further details of the experimental procedure. In each experiment the results were compared to the duplicate assays using XTT. The selected WST dyes showed exactly comparable activity to XTT. With high levels of superoxide generation (susceptible variety) the WST dyes showed slightly higher levels of detection by up to 15%, though at low levels values were identical to XTT.

Figure 1.6 Performance of WST dyes in standard superoxide assays.

a) WST-1



b) WST-8



Conclusions: Several WST dyes could substitute for XTT in the assay chemistry but had no distinct advantages over XTT. WST-1 and WST-8 were slightly more sensitive than XTT but not significant enough to justify the extra expense of their use in the manufactured kit. There were no advantages to be gained over XTT in terms of stability of solutions or solid at different temperatures. The WST dyes were much more expensive than XTT and would significantly increase the cost of the assay chemistry. Supplies of WST dyes could be a problem due to their restricted, specialist usage. XTT appears to be satisfactory by several criteria – sensitivity, stability and cost. The projected commercialization plans (see **Section 4**) and anticipated kit marketing scale made cost a prime consideration.

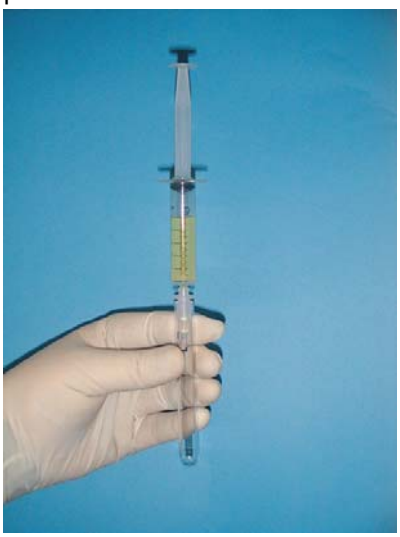
Recommendations: XTT should be adopted as the standardised detection chemistry for the diagnostic kit to minimise the costs of the diagnostic kit. Long-term storage of the tetrazolium dye solutions is only feasible at low temperatures 4^{o}C for a few days; -20^{o}C for a much longer period. For maximum stability and shelf life the solid dye used for kit manufacture should be stored in the dark at -20^{o}C or at least at 4^{o}C.

1.7 Core numbers per assay - statistics, accuracy and sensitivity: The original lab-based assays were designed around single tissue explants (~0.5-1cm cubes) and later single half cores. In discussions with the consortium scientific sub-committee this approach was regarded a) as too labour intensive for the anticipated number of tuber assays (several hundred) and b) too limited to provide statistically representative results. Assays based around the small reaction volumes (0.5-1ml) were also regarded as too awkward for inexperienced users and likely to produce inaccuracies into the results. Also a kit based on this assay would require a large number of small assay containers and would be labour intensive to dispense the small aliquots of assay solution and would be difficult to use – several hundred tubes to be assayed at precise times. In order to get around this problem it was suggested that investigations on scaling up the assay should be undertaken to accommodate multiple tissue samples per assay. This was tested initially with 5 cores (10 separate half cores) placed in a single assay container and compared to the superoxide level assay with 10 individual cores. Initially a 5ml assay volume was used. The results showed a cumulative value 22% lower than that for the individual half core assays. This was largely a

consequence of clumping of cores together causing insufficient contact between the assay solution and the surfaces of the tissue where the SO was being generated. The reagent in close contact with the tissue surfaces rapidly became exhausted diminishing the product level. To improve the mixing of reagent constant, gentle agitation was introduced on a slow orbital shaker. This greatly improved the results and the difference between single assays and cumulative, multiple assay was reduced to only 3%. To avoid the clumping of tissue cores and thus further improve the accuracy the ratio of assay solution to cores was doubled to 10ml providing an excess of the detecting solution in which all the cores were completely immersed and free to move about. With a static assay the difference between individual and cumulative assay fell to 16% and with constant agitation the difference was only 1%. The compromise between a static assay and one exposed to constant agitation, which was regarded as impractical unless end-users had access to a suitable slow shaker, was to introduce controlled agitation during the 20 minute assay period. With 2 agitations in the 20 minute incubation the difference was reduced to 10%. This level of error was likely to be acceptable considering the significant reduction in the effort required to assay large numbers of samples by using the cumulative core assay. Later studies by Fraser Milne at SAC indicated that there could be a problem arising from agitation of cores in that small starch grains or cell wall fragments were produced which could not be removed by the integral filter system in the lids of the assay bottles. Leakage of particulate material through the filters would cause erroneously high colorimeter readings (absorbance). This did not appear to be a problem during the development of the assay and subsequent trials using different filters and a range of tubers did not reveal a problem. One explanation suggested for the SAC results may have been sampling of immature tubers containing a larger proportion of small starch grains not normally encountered in mature crops.

Figure 1.7 – Reaction containers for single vs multiple cores

a) Original single half core assay with syringe filter to recover the product solution.



b) Modified multiple core assay with integral cap filter to recover the product solution.

Conclusions: The scaled up assay was considerably more convenient essentially allowing the equivalent of 10 single assays to be carried out simultaneously and providing a more statistically representative result. Mixing was always a problem to provide the tissue surfaces with fresh reagent however constant agitation while more closely representing the cumulative results of the individual assays was impractical for end users. Doubling the volume of assay solution together with limited agitation was regarded as a suitable compromise method.

Recommendations: multiple cores to be adopted as the basis for a more statistically representative assay in the diagnostic kit. Some standard agitation should be introduced during the assay to minimize the loss of accuracy. This would require to be described in the instruction manual.

1.8 Experiments on the stability and shelf life of the XTT reagent: The original report on this work has been submitted to the consortium previously in April 2007. A slightly amended version is provided here for completeness and continuity of information.

Objective: To test samples of the kit used in the most recent field trials to check if the detection chemistry (XTT) was still active as prepared by Gavin Lishman and as stored by the various industrial partners. Tests would also check for the reproducibility of the dispensing procedure used and the reproducibility of dissolution of the detection XTT prior to use. The opportunity was also taken to test sample kits which were prepared for the first set of field trials (2005) in which the XTT was > 2 years old.

Methods: Duplicate samples of the commercial kits were supplied by the participating industrial partners as indicated in Table 1. Several reports on the kits indicated that there was variable amounts or no material in the bottles so the appearance (colour) of the chemical and the approximate amount of the dried XTT chemical before dissolution was recorded by scanning the bottles on a flat bed scanner (Figure 1).

Table 1.2 – List of kit samples tested for stability of XTT activity

No.	Source	No.	Source
1	Durham (old kit) 1	**2**	Durham (old kit) 2
3	SAC 1	**4**	SAC 2
5	Greenvale 1	**6**	Greenvale 2
7	McCains 1	**8**	McCains 1
9	BPC 1	**10**	BPC 1
11	Lishman 1	**12**	Lishman 1
13	XTT standard 1.1	**14**	XTT standard 1.2
15	XTT standard 2.1	**16**	XTT standard 2.1
17	Blank 1	**18**	Blank 1
BPC – British Potato Council		SAC – Scottish Agricultural College	

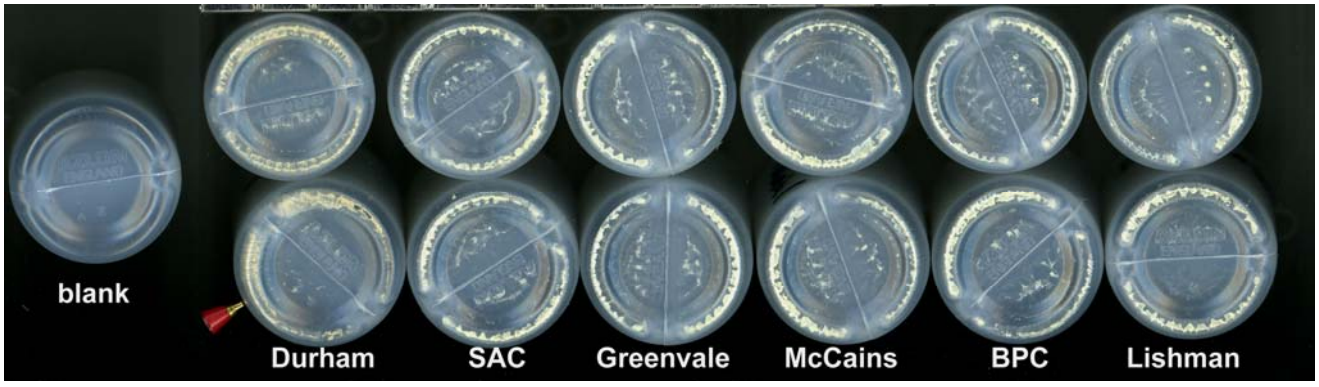


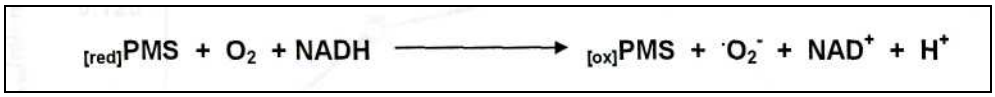
Figure 1.8 - Flat bed scan of unopened kit bottles prior to analyses

Image in **figure 5** shows a scan of the kit bottles supplied from each source alongside bottles from a previous years trial (Durham) which were ~24 months old. All bottles show the pale yellow deposits of the XTT reagent as a dry film on the bottom of the bottles. Although difficult to judge accurately the amounts do look similar and there is clearly chemical in all the bottles supplied. A slight darkening of the Durham reagent compared with the newer samples was noted. On receipt, all sample bottles were stored at 4˚C in the dark. The Durham samples had been stored throughout their shelf life at room temperature in a black poly bag in a dark cupboard.

It should be remembered that the Durham samples were prepared under conditions designed to maximise the stability of the XTT – after the XTT was dispensed and dried the bottles were gassed with nitrogen to exclude any oxygen which might cause loss of activity. The bottle lids were fitted with 'O' rings to ensure a gas-tight seal. Subsequent kit preparation excluded the gassing and fitting the 'O' ring seal.

Prior to testing the supplied kit samples, fresh XTT (supplied by Sigma) and 2 Durham samples were dissolved in 50mM potassium phosphate buffer at the required concentrations and used to check the UV and visible absorbance spectra (Figure 3 a-d).

Establishing the parameters for the assay:
Some preliminary tests were carried out to check the conditions for $^{\cdot}$**SO** superoxide generation. A non-enzymatic system of superoxide anion production was adopted utilising aerobic reaction of NADH and phenazine methosulphate (PMS) [Ewing & Janero 1997] as indicated by the following equation:



The optimum conditions of pH and reaction time had been studied previously in a Ph.D. study (S. Doherty, University of Durham 2000) and these parameters were used to establish the present assays. These experiments provided the concentrations of NADH and PMS needed to convert all of the XTT to formazan and the minimum timescale for completion of the reaction. It also provided the required formazan product for the absorbance spectrum (Figure 3d).

Solutions:
1) XTT (FW = 673.5) - Previous work estimated that the XTT needed to be > 90µM (say 100µM) for the maximal reaction within the reaction time (20 minutes). For the kit 500mg XTT was dissolved in 61.7ml methanol and then 100µl dispensed per bottle to make up 10ml of XTT aqueous solution:

500mg / 61.7ml = 810mg / 100ml 8.1mg/ml = 0.81mg/100µl=0.81mg/10ml=120µM final

The Molar Extinction coefficient for XTT formazan @ 450nm is 2.16×10^4 $M^{-1}.cm^{-1}$.
1M XTT gives an OD of 2.16×10^4
1mM XTT gives an OD of 21.6; 100µM is 2.16
1µM XTT gives an OD of 0.022

XTT for the spec assay should be the same as kits so weigh out 1.62mg XTT (accurate) and dissolve in 20ml of phosphate buffer pH7.8. (this corresponds to 120µM XTT and is enough for 20 assays).

2) NADH and PMS - The original XTT/SO assay contained 120µM XTT, 78.4µM NADH;
and 2.8µM PMS. NADH is added to the XTT and then the reaction started by adding PMS. 250µl - 1ml reaction volumes were used. Preliminary trials with the original reaction mixture (Figure 3a-b) showed that the reaction was almost complete within about 10 minutes. To ensure that excess SO was produced it was decided to compare the results of two sets of assays - i) one in which the above reaction mix was made up but with 2 x the NADH / PMS concentrations = 150µM NADH and 6µM PMS and ii) a second set in which only one tenth the amount of XTT was used in the assay with the same quantities of NADH and PMS (this represents a 20x excess over the normal reaction which goes to completion in about 20 minutes).

NADH (FW = 709.4)	21mg dissolved in 2ml phosphate buffer then 10µl added per reaction (1ml aliquots).
PMS (FW = 306.3)	5.4mg PMS dissolved in 18ml phosphate buffer then add 5µl to start reactions. The PMS should be used immediately as it is oxidised in air and turns green within about 30minutes.

3) Kit samples -10ml of phosphate buffer was added to each bottle followed by vigorous shaking and agitation to ensure complete dissolution of the XTT. This was carried out over a period of 15-30 minutes with occasional agitation. During this time none of the samples lost the filters located in the cap nozzles (*there were several reports that vigorous shaking of the bottles caused the filters to be displaced*). After dissolving the XTT the top of the cap nozzle was cut off and the XTT solution filtered into a separate tube for assay.

4) Assays - Kit samples were made up as indicated in 3). 1ml aliquots of sample solutions were dispensed into Falcon tubes and kept in the dark until assayed. A second set of 1ml samples were taken and each diluted with buffer to 10ml. 1ml of the $1/10^{th}$ dilutions were sampled into Falcon tubes and kept in the dark until assayed. As indicated above 10µl of NADH was added to each assay tube, mixed and then 5µl of PMS added, mixed and kept

in dark for 30 minutes. Sample absorbances were measured in a Beckman DU 7500 spectrophotometer. To give some idea of the colour yield produced when all of the XTT is converted by superoxide to formazan Figure 2 shows a photograph of some of full strength samples after 30 minutes (most of the colour change occurs within the first 5 minutes).

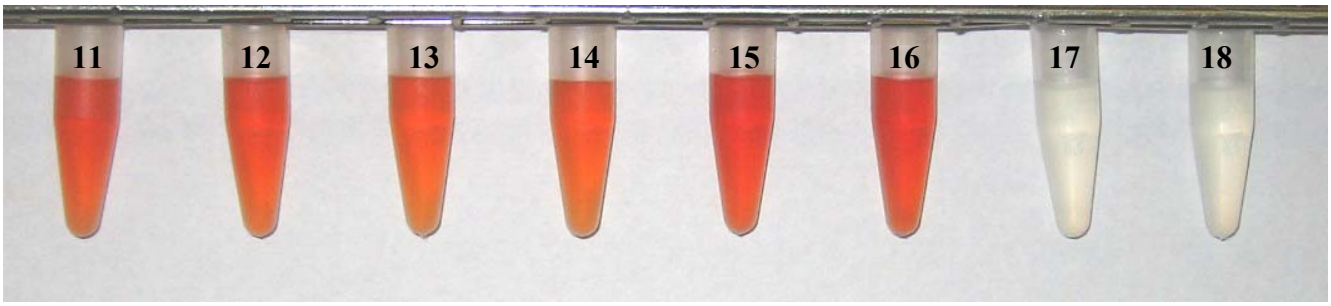


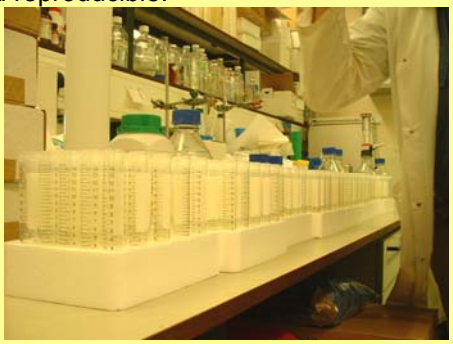
Figure 1.9 Formazan colour yield with artificial SO generator. Example XTT formazan colour yield after 30 minutes reaction with •SO using standards and kit samples (**samples 11 - 16**). The 1x concentrated starting XTT solution was almost colourless (pale yellow) (**samples 17 + 18**).

A note about the dispensing and dissolution of XTT in assay bottles

A precisely measured amount of XTT is provided as a dried (yellow) solid on the bottom of the assay bottles (See **Figure 1.8**). Comments from end-users indicated two possible problems when using this material – **a)** that there was no XTT visible in some assay bottles and **b)** that the XTT would not dissolve in the buffer by simply swirling the contents gently – some remained as a suspension. Clearly either of these problems would lead to lowered levels of XTT in the assay and inaccuracies or erroneous results. **a)** The production of the assay bottles requires manually dispensing a precise volume of XTT solution/suspension into each assay bottle (see below) and then allowing the solvent (methanol) to evaporate leaving the XTT as a dry film on the bottom of the bottle. With the exception of human error in which the odd bottle might be missed out it is unlikely that there would be many bottles supplied without the reagent. It is a possibility that the XTT film dried thinly in several bottles so that it was not clearly visible to the end user. The above tests confirm that the XTT was present in all of the assay bottles sampled and that the amount present was correct and reproducible.

Dispensing XTT into assay bottles

Gassing assay bottles with nitrogen

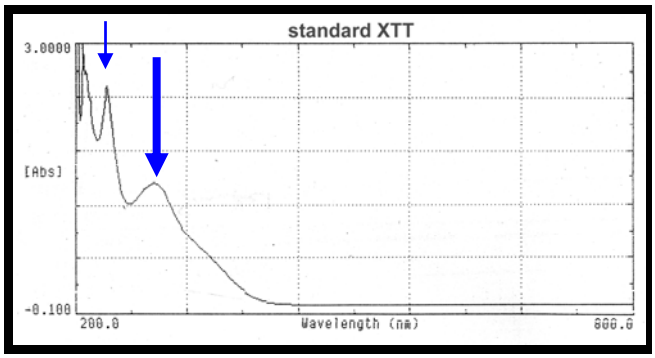


Preparing tube B, aqueous buffer

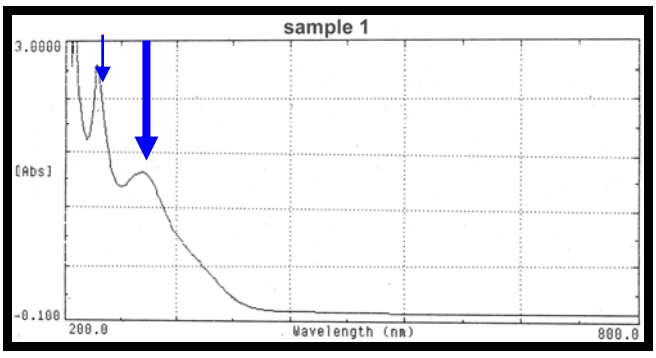
b) Tetrazolium salts are normally only sparingly soluble in water and their formazan products even less soluble (some precipitate from solution). XTT and the WST's are new generation tetrazolium salts and along with their formazan products are much more soluble. Producing assay bottles with a dried film of XTT means that the end-user has to add the aqueous buffer, as supplied, and then dissolve the XTT completely. The original instructions provided with the kits indicated that this should be done by gentle mixing for about 10 minutes. This was partly to avoid wetting the integral paper filter used for filtering the formazan product after the assay and causing problems with this step. However lab tests subsequently showed that the filters continued to work satisfactorily even after wetting and so vigorous shaking to facilitate dissolution of the XTT could be introduced. The subsequent instruction manual carried modified instructions as follows - ***Making up the assay solution*** *Remove the cap from one bottle A and one tube B. Pour ALL of solution from tube B into bottle A. Replace the cap on A and hand tighten. Dissolve the assay chemical by shaking VIGOROUSLY the contents of the bottle. Allow 5-10 minutes for the assay chemical to dissolve completely with shaking.*

Results:

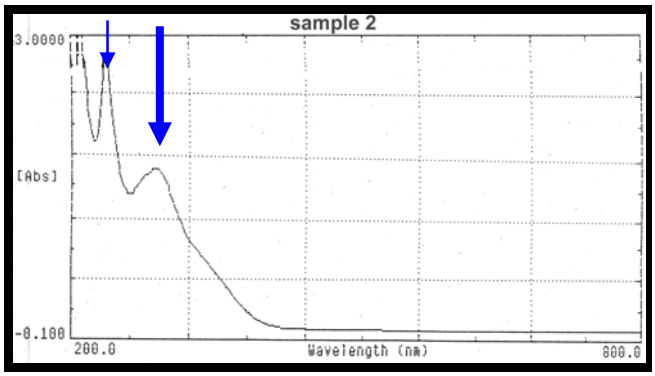
Figure 1.10 Spectrophotometric analyses of XTT and its formazan.
Standard XTT and kit sample solutions were scanned in a Beckman DU7500 diode array spectrophotometer over the wavelength range from 200nm to 800nm. The traces for the four samples scanned are shown below



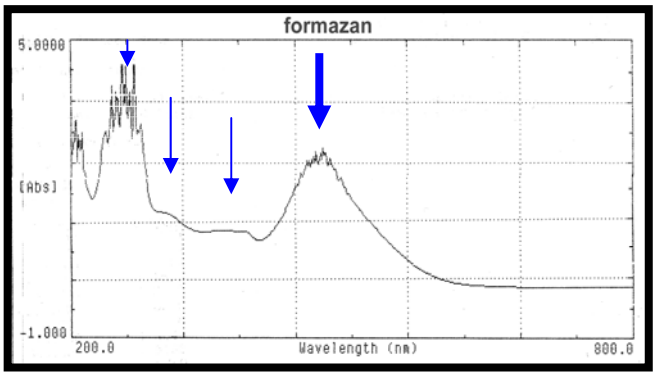
a) - freshly prepared standard solution of XT phosphate buffer



b) - XTT kit sample 1 in phosphate buffer



c) - XTT kit sample 2 in phosphate buffer



d) - formazan product after reacting XTT with excess superoxide

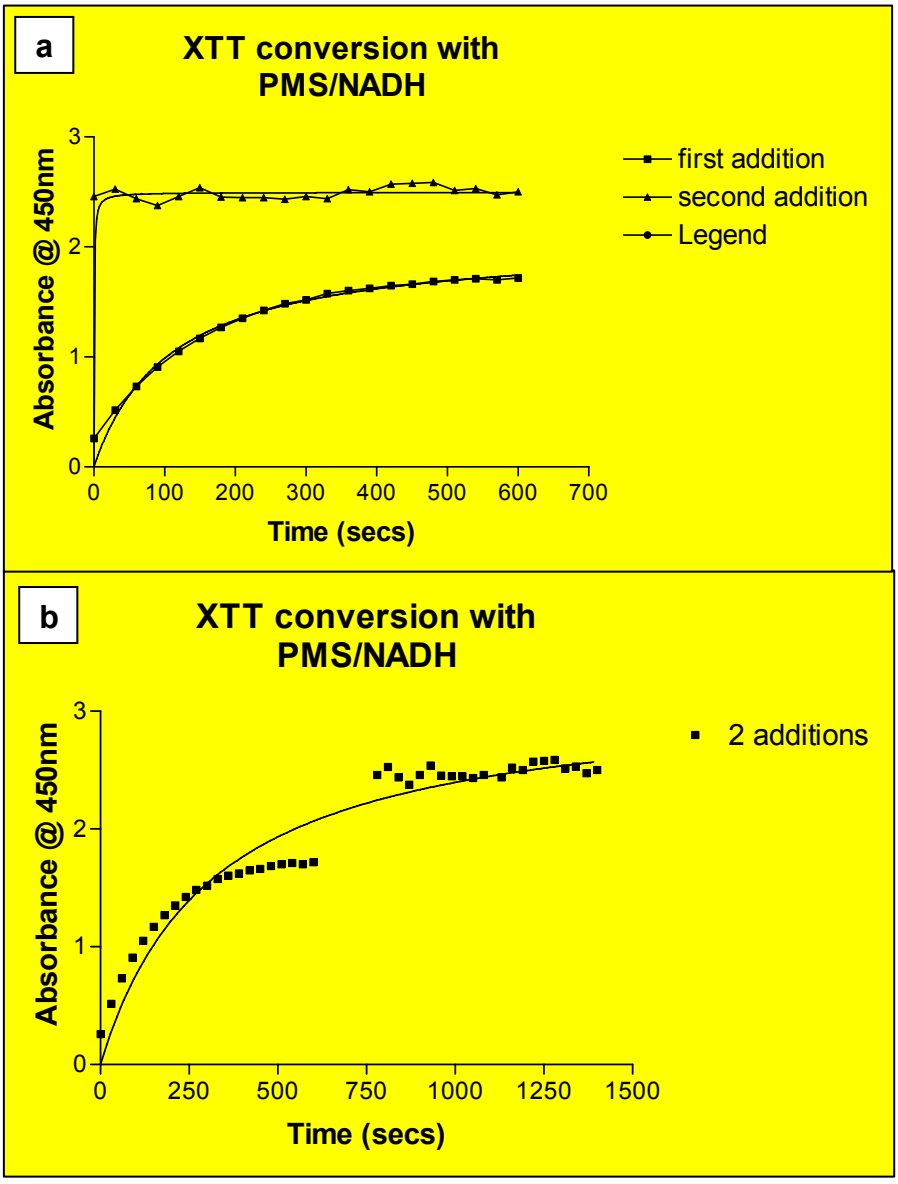
From the spectral scans of the XTT solutions before (**Figure 1.7a-c**) and after the reaction with superoxide (**Figure 1.7d**) the wavelengths of maximum absorbance can be seen for XTT at 234nm and 285nm while for the XTT formazan this changes to 450nm. Because there is almost no XTT absorbance at 450nm this is the wavelength chosen to monitor the reaction since all of the absorbance is due to the generation of formazan product. The absorbances of the formazan at other wavelengths are either minor or lie in the short UV range overlapping with those in the XTT itself so are not useful for monitoring the reaction. The two absorbance peaks in the XTT scans (234nm and 285nm) are potentially useful for checking the concentrations of the XTT reagent in solution before reacting with superoxide.

Conclusions: For these assays all samples were monitored simultaneously at the 3 wavelengths 234nm, 285nm and 450nm in a Beckman diode array spectrophotometer (DU7500).

b) Kinetics of the reaction

The progress of the original reaction mixture after 2 additions of NADH and PMS is illustrated in **Figure 1.11a)** shows the readings (at 30 sec intervals) after 1 addition and then after the second addition plotted as independent datasets. **Figure 1.11b)** is the same data but plotted on a contiguous timescale. The graphs show that a single addition is insufficient to convert all the XTT to formazan and the reaction stops after about 10 minutes. A second addition of NADH and PMS is required to convert the last XTT to formazan and the reaction reaches a stable plateau which doesn't change with further additions of NADH and PMS.

Figure 1.11 Kinetics of the conversion of XTT to formazan



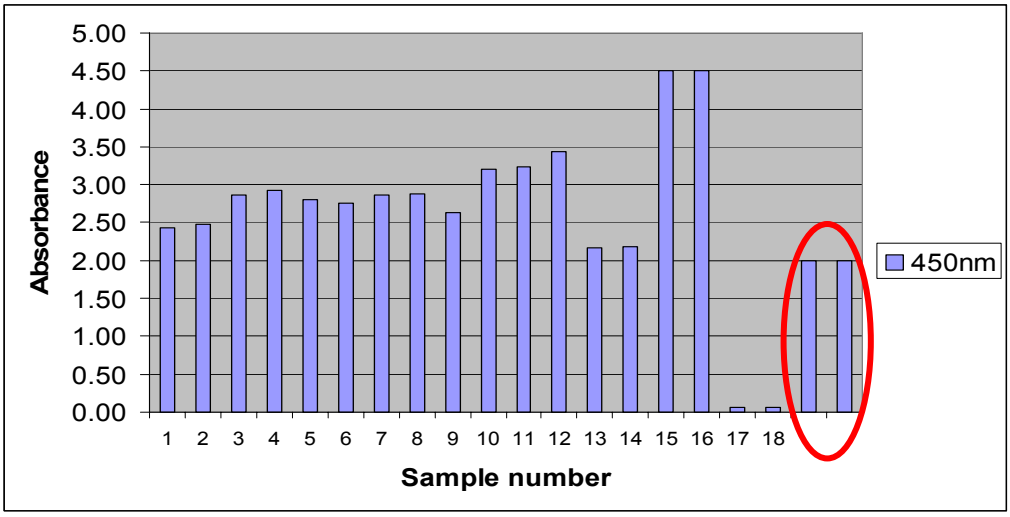
Conclusions: From these results it was decided to use 2x the original concentration of NADH and PMS and to extend the reaction time to at least 30 minutes. In addition two sets of assays were done using 1x and 0.1x the level of XTT and samples (ie 1/10th the original concentration of XTT and kit sample). In this way there would be a substantial excess of superoxide generated and all the available XTT would be converted to formazan. Furthermore spectrophotometer readings were taken for each sample at the end of the assay period (30min) and after 24h storage in the dark at room temperature to estimate the stability of the aqueous formazan product. The end points can then be compared for each of the samples and standards.

Sample assays: All samples were assayed in duplicate. **Table 1.3** lists the samples analysed. Histograms of the data were plotted (in Excel) and are presented below.

Table 1.3 – Key to XTT samples analysed

No Sample	No Sample	No Sample
1. Durham (old kit) 1	13. McCains 1	25. XTT standard 1.1
2. Durham (old kit) 2	14. McCains 2	26. XTT standard 1.2
3. Durham (old kit) 1	15. McCains 1	27. XTT standard 1.1
4. Durham (old kit) 2	16. McCains 2	28. XTT standard 1.2
5. SAC 1	17. BPC 1	29. XTT standard 2.1
6. SAC 2	18. BPC 2	30. XTT standard 2.2
7. SAC 1	19. BPC 1	31. XTT standard 2.1
8. SAC 2	20. BPC 2	32. XTT standard 2.2
9. Greenvale 1	21. Lishman 1	33. Blank 1
10. Greenvale 2	22. Lishman 2	34. Blank 2
11. Greenvale 1	23. Lishman 1	35. Blank 1
12. Greenvale 2	24. Lishman 2	36. Blank 2

Figure 1.12 Kit sample assays- variation of product absorbance with concentration
a) readings for full concentration plus an extra assay using 24h XTT solution (circled)



b) readings for 1/10th concentration XTT

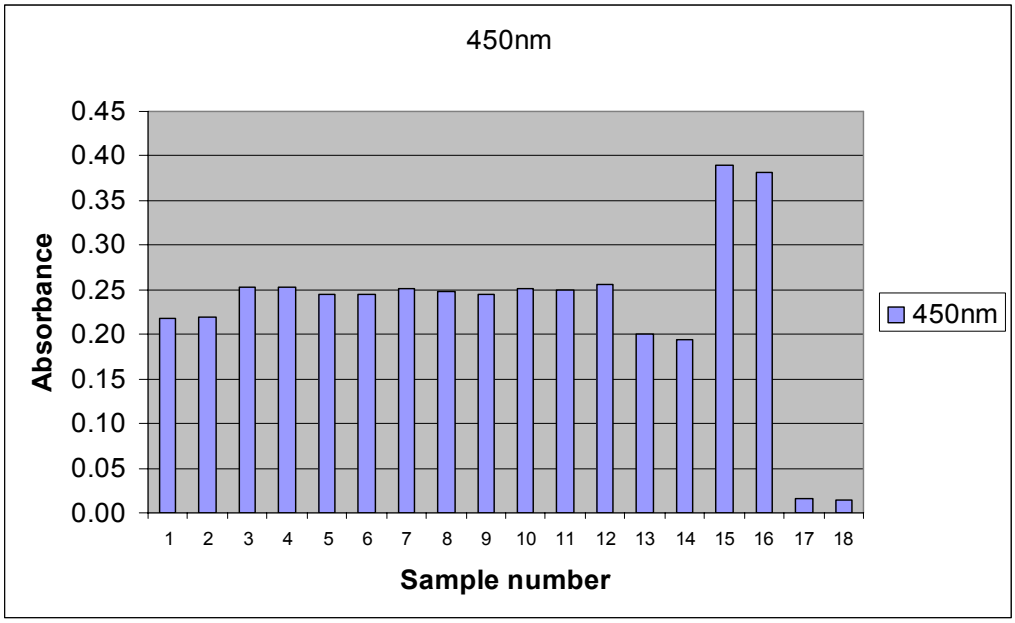
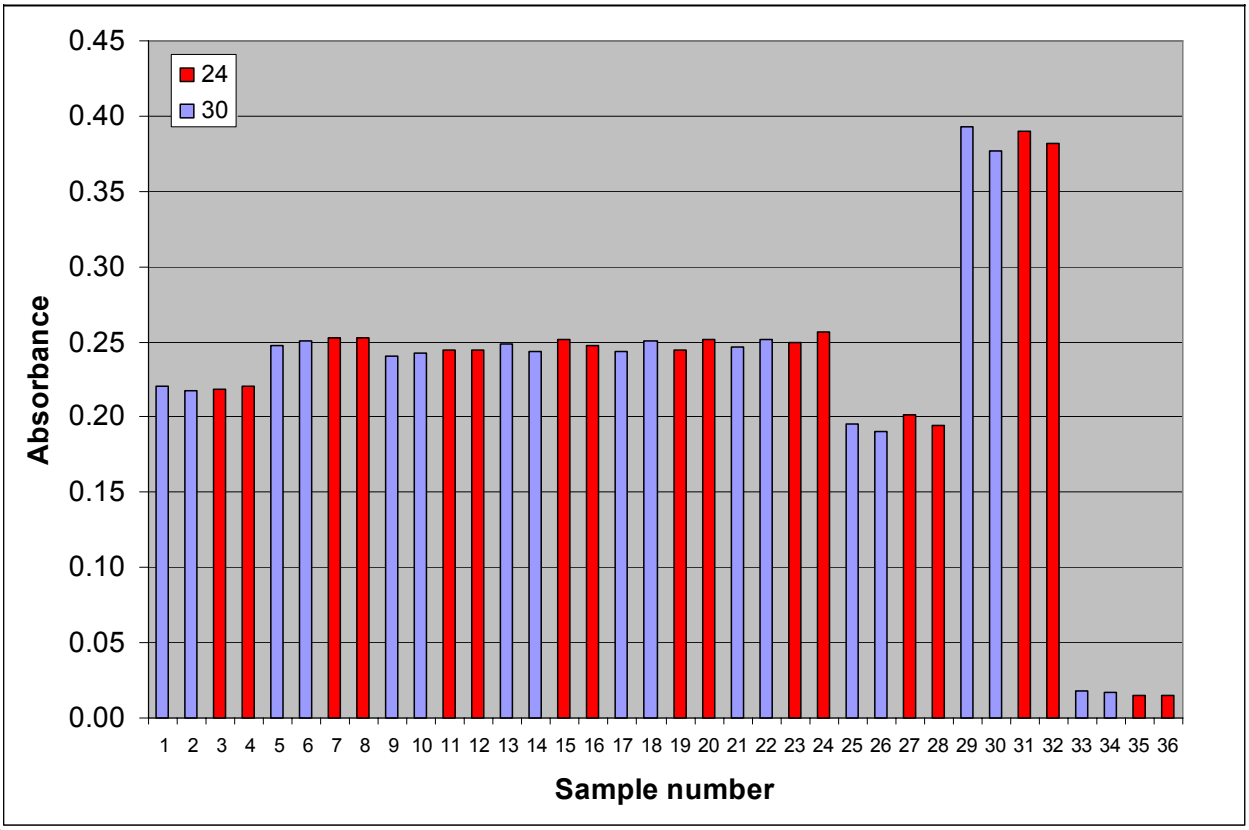
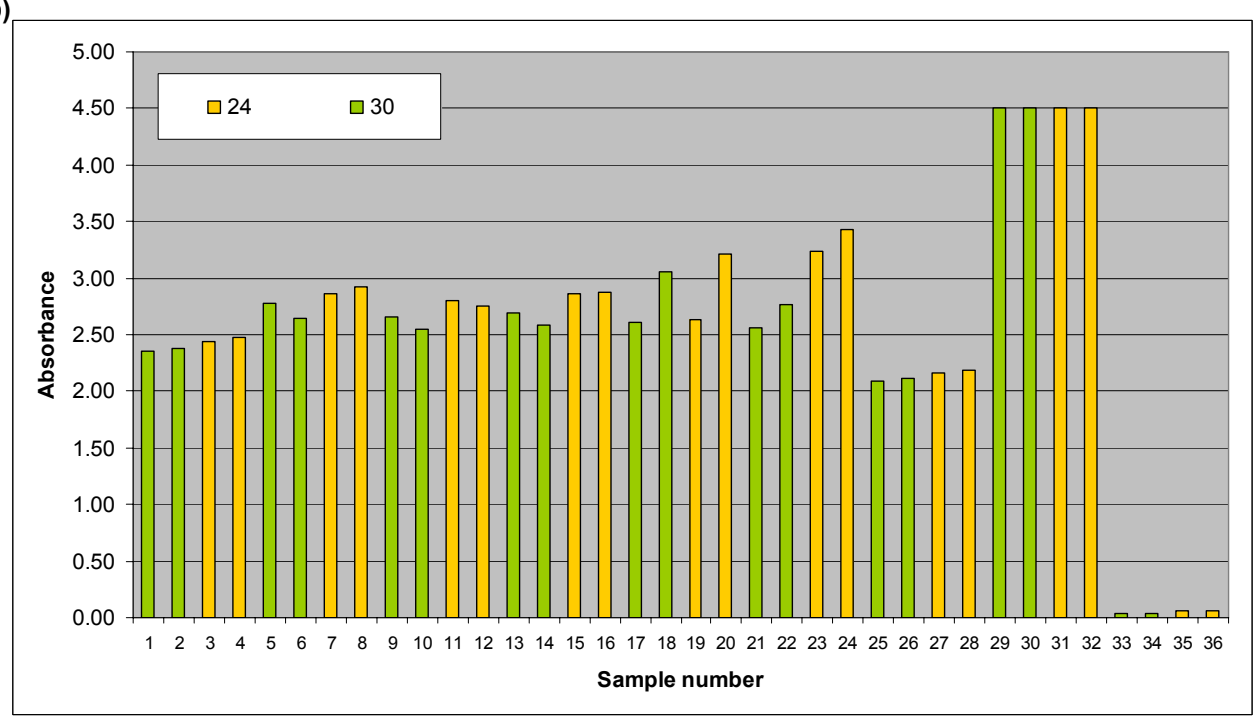


Figure 1.13 Kit sample assays – variation of product absorbance with time
a) and **b)** compare absorbances at the two concentrations after 30 minutes (blue/green bars) and after 24h (red/yellow bars) incubation in the dark at room temperature.

a)



b)



Conclusions

1) Kit samples show very similar levels of XTT in each kit bottle - the reproducibility of volume dispensing in the latest kit production is very good.

2) The differences between the kit samples (5-24) and the standard XTT solutions (29-36) arises due to inaccuracies in weighing out the small amounts of XTT required (~1-2mg). This justifies the method established to prepare the kits using an XTT solution and dispensing measured volumes which is highly reproducible.

3) The blank standards (33-36) show very little absorbance increase (even at the higher levels of superoxide generation) showing that the NADH / PMS superoxide generator does not contribute significantly to the absorbance levels at 450nm.

4) Based on Abs 285nm the XTT standards made by weighing out the solid XTT are somewhat variable due to inaccuracies in the weighing process. However, the values are reasonably close and the mean standard level is ~ the amount of XTT supplied in the kits. The Abs@285nm could be used in future to quantify the XTT in kits for quality control.

5) Abs@450nm indicates the level of formazan present in the XTT. XTT has negligible absorbance at 450nm.

6) The absorbance values of formazan produced in the undiluted (x1) XTT compared to the $1/10^{th}$ XTT dilutions are approximately 10x higher as might be expected if all the XTT is converted in both cases.

7) Comparison of results with undiluted (x1) XTT and (0.1x) $1/10^{th}$ dilutions show that the excess superoxide generation has very little or no additional effect on colour yield (formazan level) and therefore all of the XTT is converted to formazan in these assays.

8) The comparisons using both x1 and 0.1x XTT concentrations show that the colour yield after 24h incubation at RT in the dark remains almost exactly the same - the formazan product is therefore stable in this 'pure' solution (stability in the 'biological' assay may be significantly different). Potentially this means that assays could be performed on one day, filtered and then the readings of the solutions taken on the next day but this would need to be checked with a larger number of samples of different formazan content. However it is possible that` partial reactions still containing unreacted XTT might deteriorate on prolonged incubation and leading to an elevated absorbance and erroneous readings (See 13 & 14).

9) The older kit samples (1&2) show decreased level of XTT and increased background coloration.

10) The 'older' kit samples (>24 months) appear to be about 90% as active as the new ones. There is an observable decrease in the amount of formazan produced - whether this is simply caused by a variation in the amount of XTT originally or the chemical loss of XTT during long-term storage is unknown however the slight darkening of the usual XTT colour may indicate some degradation. 10% loss over two years does not seem significant and is unlikely to affect the results with tuber samples. A major concern might be if there was increased decomposition of the XTT in the presence of damp air / oxygen since the newer kit samples were not gassed with nitrogen and sealed against air.

11) Contrary to expectation XTT solutions >24h old and stored in dark still retain about 67% of the original XTT activity.

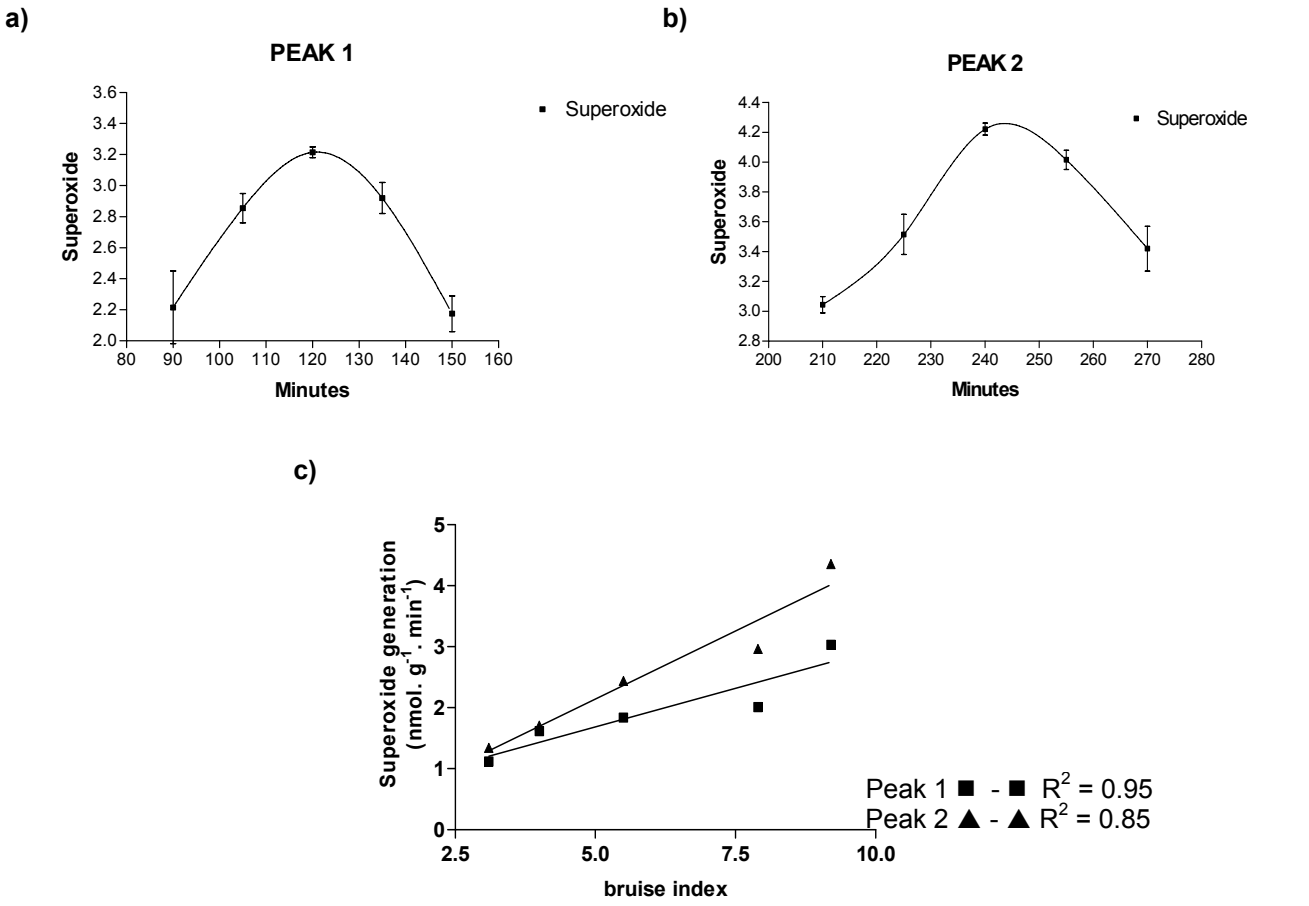
12) **Overall** there does not seem to be any problem with the quality of kit production and storage under the different conditions used by the industrial partners. From these limited tests the kits are at least as good as those produced in the lab and would be expected to perform just as well in the Blackspot detect test.

Recommendations: As far as the biochemical tests go there are no stability problems in preparing XTT for use in the kit. Estimates of the usable lifetime of the reagent bottles for multiple core assays appears to be in excess of 12 months – well beyond the anticipated shelf life required by the end users for a single growing season. It would not be recommended for end users to keep the XTT reagent bottles from one growing season to another since inappropriate storage could make assay results inaccurate. The need for gassing reagent bottles with nitrogen to displace the air and improve the activity of the XTT in the kit could not be justified on the basis of the above results.

1.9 Correlations at different times after impact: The original work on the response of susceptible tuber tissue to impact showed a distinctive biphasic (2-peaks) generation of superoxide radicals (**Figure 1.6**) in which an initial peak of activity was displayed after a lag period of about 1-2h post impact. This was followed by a decrease and then a second increase in superoxide generation after about 4h post impact (Johnson *et al* 2003). Generally the second peak in addition to being longer from the time of impact was a larger peak with more SO being generated. Whilst the consortium was considering the field use of the kit based on experience with the initial kits the question arose as to the timing between impacting harvested tubers and performing the SO assays. Concerns were raised that time was extremely short for sampling large numbers of tubers from the field, impacting these tubers and then performing the SO assay exactly 2h after impact. The question was also posed as to how stable the product absorbance would be if colorimeter readings were delayed during the testing. It was reported that performing the test on the recommended numbers of tubers required a sustained effort and prolonged impacting, coring and assay. Adhering to the strict timescale dictated by the assay was very difficult and an indication of how long the assay end result was stable a) in the presence of the tissue cores and b) after the cores had been removed. It was clear that for as long as the tissue remains in contact with the solution conversion of XTT to its formazan would continue until the tissue radical generation response diminishes – in excess of 4h according to the kinetics of the response studied earlier. Clearly the radical-bruise correlation would have been lost if the 20 minute used to calibrate the assay was exceeded. Experiments performed in the previous section (**Part 1.8**) showed clearly that once separated from the source of the free radicals (i.e. the tissue cores) the formazan product was completely stable for at least 24h if kept in the dark.

The question also arose as to whether the assay could be performed 4h after impacting the tubers thereby exploiting the biphasic response reported by Johnson *et al* (2003). Would the correlation between SO generation and bruise susceptibility be maintained and would there be any loss of sensitivity. The results are shown in **Figure 1.14** in which the SO generation across peaks 1 (**a**) and peak 2 (**b**) are shown and the resultant correlations with the bruise index.

Figure 1.14 - Correlations between superoxide levels generated post-impact after 2h and 4h.



The Pearson R^2 values for peak 1 was 0.95 and for peak 2 0.85. The lower Pearson coefficient for peak 2 indicated a poorer correlation with higher variability. Variability in timing post impact will have an effect on bruise index. The effect would be up to 1 bruise index unit per 15 minutes. Variability in readings increases away from the point of maximum SO generation.

Conclusions: The linear correlation between level of SO generation and bruise susceptibility observed 2h after impact (■-■)is maintained at 4h after impact (▲-▲). The level of SO generated is greater at this time giving a steeper gradient and a more sensitive assay but the variability of the estimated values is greater. Because of the increased time period there is probably a greater error in targeting the second peak of SO generation and results may therefore be more variable. Using the second superoxide peak causes a reduction in accuracy of about 12% which would mean the kit will be 85% accurate, compared to 95%.

Recommendations: Unless there is good reason to change from the standard two hour period from impact to assay this should be maintained for the sake of accuracy and reproducibility. Changing the time would also mean that the colorimeter calibration and visual comparison charts would need to be re-calibrated. An option for two time periods (2h or 4h) would be difficult to implement and could be confusing for end-users.

1.10 Risk assessments and evaluation

There are two chemical components in the kit – a standard potassium phosphate buffer of neutral pH and the tetrazolium salt which is dissolved in the buffer just prior to use. The buffer contains only potassium phosphate salts and poses no hazard. The tetrazolium salts used in this study comprise XTT or any of the newly synthesized WST's. The following is a compilation of relevant safety statements and recommendations taken from a number of authoritative and commercial sources. The MSDS for WST-1 salt is taken to be representative of the WST properties since these dyes are all closely similar. Additional MSDS data is provided in **Appendix 4** supplied on the CD.

Product Name: XTT
Synonyms: XTT Sodium Salt
Appearance/Odour: Yellow Solid with no odour.

XTT may be irritating to skin, eyes, and upper respiratory tract. It may be harmful if swallowed, inhaled, or absorbed through skin. The toxicological properties of this material have not been fully investigated. To the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated. XTT is toxic and may cause heritable genetic defects.
Routes of Exposure: Eye contact, skin contact, inhalation and ingestion
Eye: Direct contact may cause mild acidic irritation, redness, tearing, and blurred vision.
Skin: Prolonged exposure may cause minor acidic skin irritation. May be absorbed through the skin.
Ingestion: If ingested, this material may cause irritation to the mouth, throat and gastrointestinal tract. May be harmful if swallowed.
Inhalation: This material may cause irritation to the respiratory tract including injury or illness.

Handling precautions: recommend the use of gloves, lab coats, and eye protection (safety glasses while using any of these chemical reagents). In case of contact, immediately wash skin with soap and copious amounts of water for at least 15 minutes. In case of eye contact, immediately flush eyes with copious amounts of water. If swallowed, wash out mouth with water provided person is conscious. If inhaled, remove to fresh air. If not breathing give artificial respiration. If breathing is difficult, give oxygen. In case of contact, immediately flush eyes with copious amounts of water. Call a physician.
Acute effects (ND=not determined)***:***
May cause skin irritation. Skin Irritation: ND
May be harmful if absorbed through the skin. Dermal LD50: ND
May cause eye irritation. Eye Irritation : ND
Material may be irritating to mucous membranes and upper respiratory tract.
May be harmful if swallowed. Oral LD50: No oral LD50 in rats information is available.
May be harmful by inhalation, ingestion, or skin absorption. Inhalation: ND
Sensitization: ND
***References*:** **MD Biosciences** http://www.mdbiosciences.com
Trevigen, Inc. http://www.trevigen.com
Research Organics http://www.resorg.com

Product Name: WST-1 (as representative of all the water soluble tetrazolium salts used in the project)
Synonyms: 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt.
Appearance/Odour: Slightly yellowish brown powder, no odour.

Identity: WST-1
Section 1. Chemical Identification
Trade Name: WST-1
Manufacturer: Dojindo Laboratories
Address: Kumamoto Techno Research Park, Tabaru 2025-5, Kamimashiki-gun, Kumamoto 861-2202, JAPAN
Telephone: +81-96-286-1515
Data prepared: December 25, 2000
Section 2. Composition/ Information on Ingredients
Chemical Name: 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,monosodium salt
CAS Registry #: 150849-52-8
Section 3. Health Hazard Data
Route(s) of Entry:
Inhalation? Yes; Skin? Yes; Ingestion? Yes
Health Hazards (Acute and Chronic): May be harmful by inhalation, ingestion or skin absorption.
Causes eye and skin irritation. The material is irritating to mucous membranes and upper respiratory tract.
Signs and Symptoms of Exposure: Not available
Medical Conditions Generally Aggravated by Exposure: Not available
Section 4. First Aid Procedure
In case of contact with eyes, immediately flush eyes with copious amounts of water for at least 15 minutes. In case of contact, immediately wash skin with soap and copious amounts of water. Assure adequate flushing of the eyes by separating the eyelids with fingers. If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. If ingested, wash out mouth with water. Call physician. Discard contaminated clothing and shoes.
Section 5. Fire and Explosion Hazard Data
Extinguishing Media: Carbon dioxide, dry chemical powder, foam and water
Special Fire Fighting Procedures: Wear protective clothing, respirator, chemical safety goggles, rubber boots and heavy rubber gloves. Fire may produce irritating or poisonous gases.
Section 6. Other Precautions
Store in cool (0-5 ^{o}C) and dry place.
***References*:** Dojindo Laboratories http://www.dojindo.com/

The hardware in the kit poses relatively low risk if these are used correctly. The impacter could cause harm if directed onto head or limbs. The final impacter design has a sharp cutting ring and the corer contains very sharp cutting blades – these devices should be handled with care avoiding contact between the cutting surfaces and fingers and hands. Appropriate safety phrases should be inserted into the instruction manual as indicated below.

Conclusions: The chemicals included in the kit for the detection of superoxide are not unduly toxic but contact with any of the tetrazolium dyes (XTT, WST's) should be avoided. Because these are relatively novel chemicals much of the safety data is not yet available especially concerning any hazards associated with long term exposure. In this respect measures should be adopted which minimize any exposure to the tetrazolium dyes. Ethanol/methanol solutions of the dyes are highly flammable and appropriate precautions taken including handling these in a well ventilated room and in the absence of any naked flames; the solutions are highly concentrated so for the preparation of the kits disposable gloves and safety glasses should be worn. End users should avoid contact with the aqueous solution – wash contaminated skin with copious water; in the event of contact with the eyes wash with sterile eyewash and seek medical advice as soon as possible. The only other chemical component in the kit is a standard, buffered solution of potassium phosphate which has little or no toxic effects.

Recommendations: Safety information and guidelines to be included in the **kit instruction manual** (**See Appendix 1**) as follows **Safety Considerations**: **Chemical** The test solution is a weak solution of 2,3 – Bis (2 – methoxy – 4 – nitro – 5 - sulphophenyl) - 2H – tetrazolium – 5 - carboxanilide in a phosphate buffered solution. Avoid skin and eye contact. In case of skin or eye contact rinse with plenty of water for 15 minutes and seek medical advice. **Physical** The impactor has sharp pins on the barrel end. It fires a bolt at high speed. UNDER NO CIRCUMSTANCES SHOULD THE IMPACTOR BE FIRED AT ANYTHING OTHER THAN POTATO TUBERS. DO NOT FIRE IN THE AIR AS THIS WILL CAUSE DAMAGE TO THE DEVICE. The corer contains razor sharp blades. Take care of fingers and hands.

Part 2 - Studies relating to the development of the hardware components of kit

Objective 2 – Hardware development

Task 6 – Design and build prototype 2

Task 7- industrial evaluation of prototype 2

Task 8 – Re-design and build final prototype

Task 9 – Evaluation of final prototype

Objective 4 – Commercial exploitation

Task 16 – assess performance of prototype relative to market needs

Rask 17 – design for manufacture

The development of the impacter / corer and initial impacter evaluations were mainly carried out at Durham. Field evaluations were carried out by several consortium members in numerous field trials including several after the Link project period was finished. Subsequent development and construction of the final prototypes of impacter ('bruiser') and corer was carried out by Martin Lishman Ltd with some quantitative evaluation at Durham and field evaluation by consortium members. The design and construction of the prototypes was more involved than originally anticipated with a total of 5 impacter prototypes and

2.1 Introduction: There are three main hardware components of the diagnostic kit – impacter, coring device and colorimeter. Together these are the most expensive items to manufacture and provide in the kit but were anticipated to be re-usable from one growing season to the next. By the end of the previous programme prototype impacter and corer devices had been designed and built and were used in many of the bruise correlation and testing experiments. **Figures 2.1** and **2.2** illustrate many of these devices, were abstracted from the previous BPC and Defra-funded project reports: "*A Novel and Rapid Diagnostic System for The Prediction of Tuber Susceptibility to Blackspot Bruising in the Field*".

Figure 2.1 – The original design of impacter. Gun shaped impacter design in place at the commencement of this project

One of the major objectives of the LINK programme was to design and build improved prototypes of the original impacter and coring devices needed for the diagnostic kit (**Objective 2, milestones 3 and 6**). The following aspects were considered in the design and construction of the impacter devices following detailed discussions with the consortium members and from the results of evaluations from lab and field testing.

2.2 Impacter design - consortium criteria for 'field use' design:

Functionality – impacter is required to meet the needs of the diagnostic kit and to replace the less convenient systems designed for bruise study methods (falling bolt, hot/bruise barrel, swinging pendulum). It is important to appreciate that these impacter designs were not directed towards producing an alternative 'bruising device' *per se* but rather to produce an impacting device which would provide enough force on the tuber tissues (cells) to initiate superoxide generation symptomatic of mechanical damage. The practicality of the convenient impacting device has led subsequently to its widespread use a bruising device (*hence the name 'bruiser' coined for the production prototype*) and could prove to be a valuable tool in its own right.

Subsequent research (see later) showed that the two phenomena (superoxide production and bruise pigment production) could be separated simply by adjusting the level of energy delivered by the impacter. This is analogous to the separation of tuber tissue cracking damage and bruise pigment production by adjusting the level of energy input.

- **Economical** – impacter was required to be cheap to produce (due to the relatively small numbers anticipated to be required this was not deemed to be a major consideration).

- **Simple** – design required to be simple in order to facilitate initial construction and subsequent repair, re-calibration and cleaning. From the start it was never intended that the end-user would be required to carry out these latter operations but rather this would be a 'return-to-base' exercise for cleaning, repairing and re-calibration.

- **Reliable** – required to deliver reproducible impact force with multiple impacts and over a prolonged period. Anodising the aluminium impacters was included in the earlier models (Mks 2-4) to improve the smoothness of the internal surfaces of the barrel and to provide a measure of protection since aluminium corrodes easily in contact with water and salts. Some of the field tested impacters showed some signs of corrosion when returned for re-evaluation.

- **Ergonomic** – required to be convenient and easy to use over a prolonged period. Field testing subsequently verified that the design would need to be comfortable for the user during the impacting of hundreds of tuber samples over a period of several hours / day and over several days during the growing season.

- **Transportable** – it was invisaged that impacters may be required to be carried from site to site possibly by commercial airlines especially by industrial partners with international divisions. Thus redesign of the original 'gun' shape (**Figure 2.1**) was considered a major and early consideration although this was only raised after the Mk 2 (prototype 2) design was completed. Interestingly the original patent covering the diagnostic kit components (METHOD AND APPARATUS FOR DETECTING RESPONSE TO DAMAGE AND DIAGNOSTIC METHOD THEREFOR) suggested a vertical spring-loaded impacter design (See figure above) rather than the initial gun-shaped device which the engineers.

- **Adjustable** – as became apparent during some stages of the project a degree of adjustability was useful for the lab and initial field testing impacters. This allowed fine adjustments to the force of impact delivered. Adjustment would have been useful to counter variation in the spring elasticity encountered in some of the later prototypes. However this was deemed an unnecessary complication and expense and was deleted from the plans.

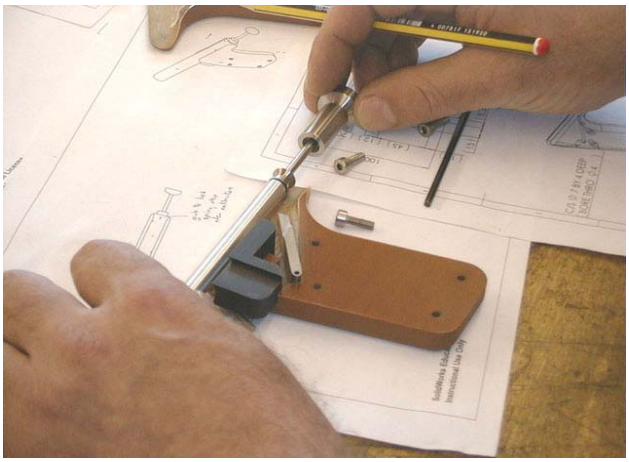
- **Mechanics of force delivery** - a key feature for consideration was the radius of the impacter head. This is critical because the energy delivered from the spring through the sliding shaft to the head is channeled through the head and is dispersed through the tuber tissue on impact. If the head is too convex (sharp) this would lead to a narrow cylinder of tissue, severe tissue compression (pitting) and a high impact force. Too flat a head would lead to maximum dispersion of the force of impact especially since the tuber itself is slightly convex. The latter aspect would make reproducible impacts difficult and subjective. To optimize the head radius a set of experiments was performed using a series of heads constructed with different radii.

2.3 Impacter design – design and construction of impacter prototypes:

Figure 2.2 – The Mk 2 prototype based on designs proposed at the end of the previous programme *(HP0217) A NOVEL AND RAPID DIAGNOSTIC SYSTEM FOR THE PREDICTION OF TUBER SUSCEPTIBILITY TO BLACKSPOT BRUISING IN THE FIELD*

a)

b)



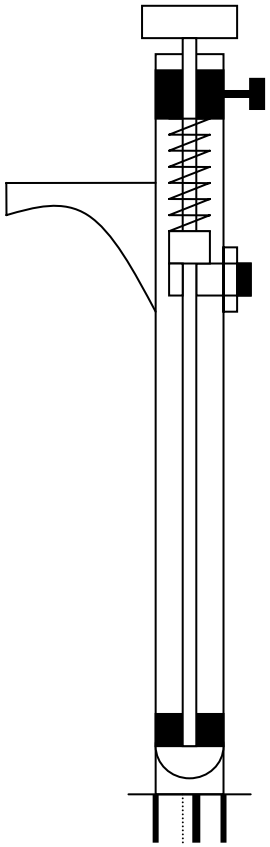
c)

d)

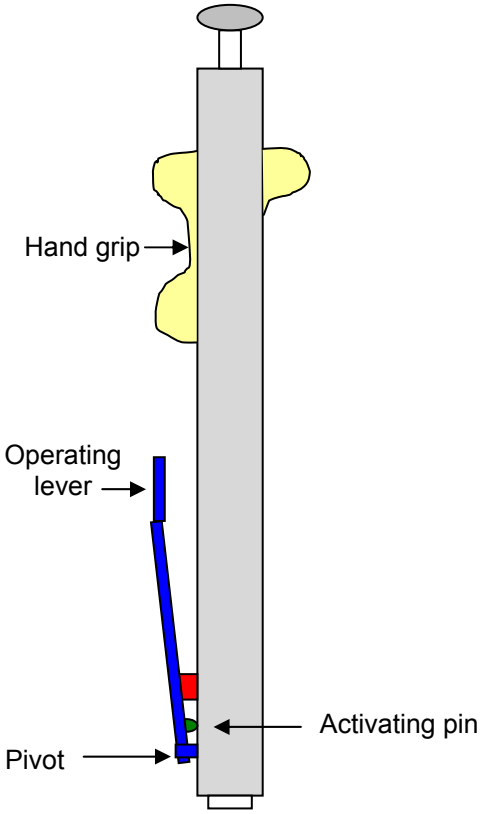
Figure 2.3 shows different stages in the manufacture of the second prototype impacter (Mk 2), the first of four impacter prototypes constructed during this programme. **a)** virtual impacter imaged in AutoCad. Impacter was designed and built by the staff of the physics engineering workshop, University of Durham. **b)** assembly of the trigger mechanism and plunger cocking system. **c)** the finished impacter prior to anodising to protect the aluminium surfaces **d)** the finished impacter in use for delivery of a precise impact force. Three impacters of this design were produced, tested using the Kistler link force load cell system described below (**Figure 2.6**) and used for preliminary 'in field' testing by the consortium partners. Feedback from these trials guided the prototype designs for the subsequent impacters. The Mk 2 was followed shortly by the Mk 3 (blue) impacter which was the first design to move away from the gun-shape following early suggestions by the consortium management committee. The Mk 3 was produced in sufficient quantities for the consortium partners to keep and carry out more extensive field trials. The Mk4 (gold) impacter was designed to address some of the ergonomic and operational problems encountered in the Mk 3 model and again was built in sufficient numbers (12) for the consortium partners to conduct extensive field trials. The Mk3 and 4 impacters were designed and built at the University of Durham by the physics engineering workshop. The final pre-production prototype, the Mk 5, was designed and constructed by Martin Lishman Ltd. incorporating the final improvements suggested by the consortium end-users. The Mk 5 was renamed the '*Lishman Bruiser*' as a more practical name than '*Impacter*' in preparation for commercial production of this device.

Figure 2.3 - Alternative designs and descriptions suggested by consortium for a more stable handling, cylindrical impacter (the Mk 3)

a) De-soldering device suggested by *S.Johnson* as a possible housing for impacting mechanism



b) Schematic showing anticipated components to meet the specifications – submitted by unknown.



c) Design submitted by *P. Harkett* (McCains,GB Ltd.)

COCKING HANDLE : Prefer an ergonomically designed handle with hand/finger grips

ADJUSTER : Need locking mechanism and screw adjustment of spring tension. Range of adjustment?

SPRING : As prototype impacter. What is spring reproducibility?

GRIP : A loop or a hook, need optimal position to counter-balance push-button.

BUTTON & YOKE : Mechanism as per desoldering device, with indented shaft and yoke triggering mechanism. Change position?

BARREL : Diameter and length as before.

SHAFT : As before

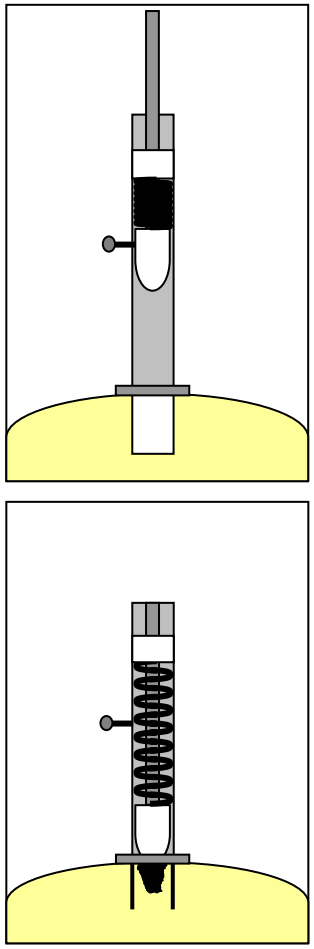
BUSH : Retain to aid friction and keep barrel clean.

HEAD : Radius either 15 or 20, to be confirmed by experiments (see later).

FLANGE : Not sure whether this part required?

LOCATING FORKS : Tapered, 3-4 forks, depth 2-2.5mm. Fitting?

d) Further suggestions from consortium members collated from various meetings and discussions.



e) *Original patent design impacter in primed state (top) and relaxed (bottom). *R.Croy*

Figure 2.4 – Mk 3 and 4 prototype cylindrical impacters. Main changes between these prototypes was the change in the position of the firing button higher up the barrel – easier and more stable in use.

Figure 2.5 – evolution of the impacter design during the project. 12 of the Mk 4 prototypes were manufactured in Durham for evaluation in field trials. This model rectified all the design issues raised by the consortium based on trials with the Mk 3 prototype. The final version (Mk 5) – described later (**Part 2.12**) was manufactured by Martin Lishman Ltd. and incorporated all the improvements suggested by the detailed 'in-use' evaluations during field trials. Impacter Mks 1, 2 and 3 were adjustable by various means while Mks 4 and 5 were not made to be adjustable so relied on uniform quality pre-defined spring tension for reproducible performance..

a) original falling bolt

b) original Mk 1 prototype

c) Mk 2 'gun-type' prototype

d) Mk 3 prototype (first cylindrical impacter)

e) Mk 4 prototype (field trial evaluations)

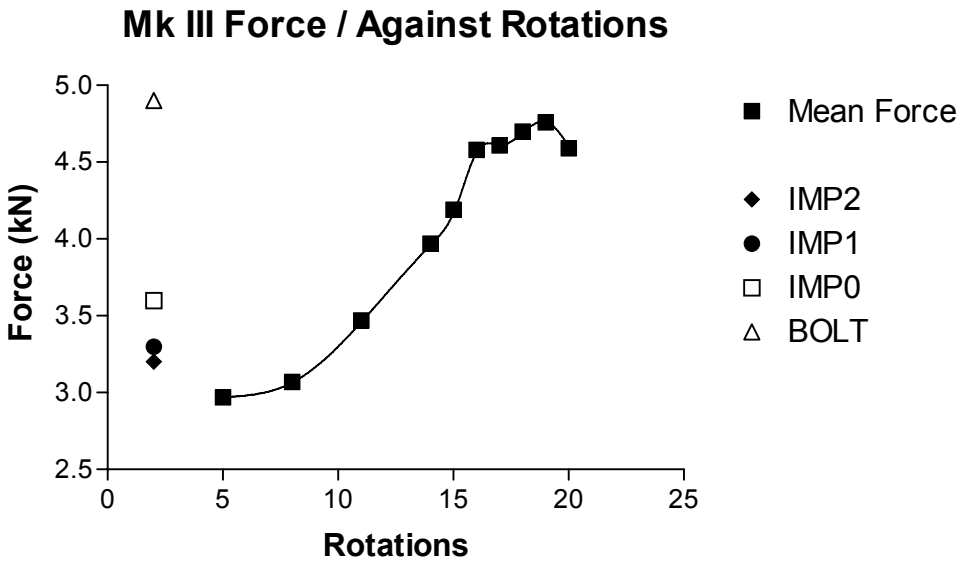
f) Final pre-production prototype Mk 5 (See **section 2.12**)

2.4 The advantage of adjustable impacter models

Impacter models Mk 1 – 3 were all made adjustable to allow fine adjustments to the internal spring providing the force of impact. In this way the same impacter could be used to deliver a range of different forces (energies). Subsequently the adjustment facility was removed from the later designs as a cost saving measure and a safeguard to prevent end-user alteration of the calibrated impact delivery. However it is worth noting the advantage that this adjustment has over non-adjustable models by way of an experiment in which the impact force (mean of multiple impact forces) was plotted against the adjuster settings in a Mk3 impacter. The results plotted in **Figure 2.7** shows clearly the fine regulation in force delivered using small changes to the adjuster setting (rotations). Depending on the properties of the spring this allows an

adjustment in the range of forces delivered up to 150% in a single impacter. Beyond a certain degree of adjustment and compression of the spring there is little or no increase in the force delivered, a consequence of the physical properties of the spring.

Figure 2.6 - Variation in force of impact using the Mk 3 impacter with spring compression adjuster



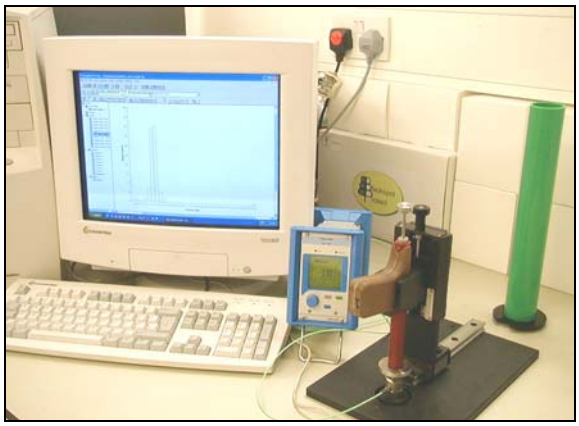
Conclusions: Adjustable impacters are useful in situations where a range of impact forces are required to test tubers. This design also makes it easy to fix the impacter to deliver a precise impact force during routine maintenance. The adjustable impacters are however more difficult and expensive to engineer. Settings (rotations of adjuster) and force are not linearly related so would not be suitable for end-user adjustment. Adjustments to a precise force would require the force link load cell and charge meter apparatus to check and finely adjust the settings.
Recommendations: Unless the subsequent impacter designs do not produce a device capable of delivering a precise and reproducible force there is no point in producing an adjustable design which would cost more. An adjustable impacter would be more versatile and could be useful for testing different types of physical damage – shatter, cracking as well as bruising – by increasing the force of impact. However further testing would need to be undertaken to ensure that the range of forces that could be selected would impart the appropriate damage.

2.5 Impacter design – quality control of performance:

All the impacters except the final prototype (Mk5) plus the impacter test rig and associated equipment were designed and constructed in collaboration with the Department of Physics, University of Durham.
The impacter design comprises of a spring-loaded plunger which delivers an impact to the surface of the sample tuber. The energy imparted to the tuber surface is then dissipated through the subdermal tissues causing damage to the constituent cells. The level of damage incurred and the resultant responses (radical generation and bruise development) is dependent on the physical and mechanical properties of the tuber tissues. The point of impact was essential to record accurately in order to be able to sample the underlying damaged tissue to assess. During the design of the impacters various means to mark the position on the tuber surface were devised considering clarity and accuracy of marking as well as minimizing non-impact related damage (See **Part 2.13** below).

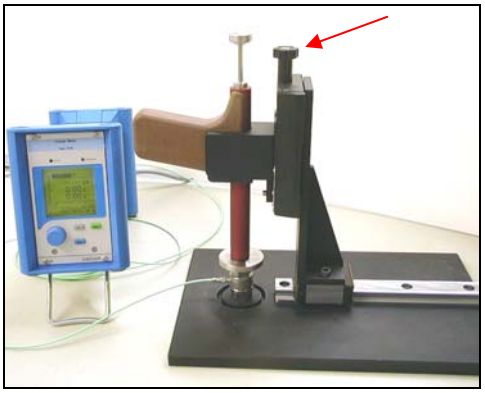
Figure 2.7 – Instrumentation for the evaluation of impacter performance and reproducibility. This equipment was also used to assess the reproducibility of spring performance by testing each spring independently, inserted into a single impacter. In the later stages of the project the test gear was transferred to Martin Lishman for quality control of the manufactured impacters.

a)



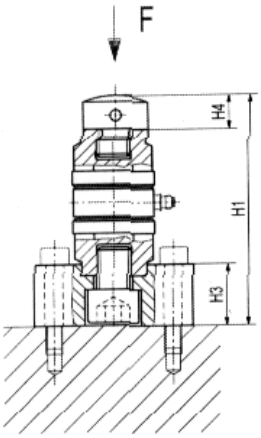
All data on force measurements were recorded and transferred to *Graphpad Prism* or *Excel* spreadsheets for statistical analysis and plotting.

b)

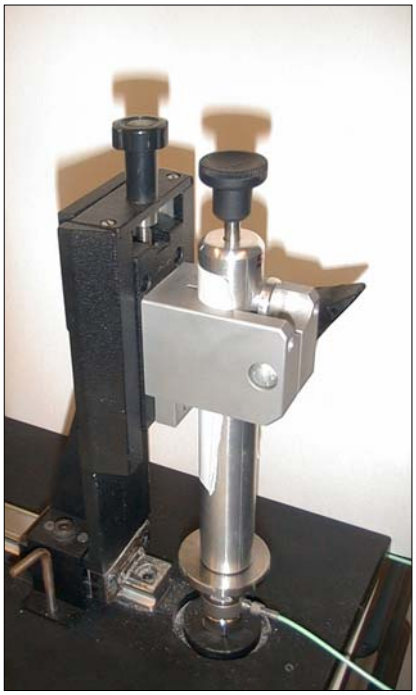


A Mk 2 impacter mounted on the force link load cell connected to the Kistler charge meter. The load cell is protected from direct impact by a steel impact plate. Fine adjustments of the height of the impacter barrel above the load cell impact plate are made using the adjuster (arrowed).

c) *Kistler* force link load cell as fitted to the impacter clamp stand and connected to the charge meter. A steel impact plate sits on top of the load cell; F indicates the direction of the impacter force.



d) A Mk 5 impacter held in a modified screw clamp device undergoing evaluation.



Critical to the function of the impacter in the diagnostic kit is the reproducibility of the impact force mechanism. This relies on the supply of consistent quality (elasticity) of springs and the free movement of the impacter head within the barrel of the impacter. The assembled impacter prototypes were evaluated using an apparatus assembled in Durham as illustrated in **Figure 2.7**. This comprised of a kistler force link (ceramic load cell) and data capture system comprising of Kistler charge meter model 5015 with direct LCD display of measurand, maximum, minimum and mean values. The instrument has fully menu-guided operation. Clamping systems to immobilise the various impacter prototypes, load cell pressure/impact plate and stand were all constructed at Durham. The method for testing was devised at Durham and a detailed

instruction booklet provided (See **appendix 2**). Subsequently the Kistler charge meter, load cell and impacter clamps were all transferred to Martin Lishman Ltd for the quality control of the manufactured impacters.

The following sections describe the methods of assessment of the impacters. The method essentially involved mounting the test impacter securely in its specific clamp and then finely positioned using the adjustment screws so that the impact head but not the end of the barrel rested on the stainless steel impact plate. The impacter was then primed and fired an appropriate number of times and the resultant reading for the force of impact recorded from the charge meter instrument. Depending on the required level of testing between 20 and 100 impact forces were measured for each impacter. A step-by-step detailed account of the impacter testing procedure and the subsequent data processing is provided in the accompanying **Appendix 2** - **Impacter testing Instructions**. This method of evaluation was capable of picking out any impacters which had a fault affecting the force delivered or the reproducibility of the impacts. Early in the development of the impacters several were found to have minor flaws which once detected could be fixed relatively easily. The majority of the impacters performed satisfactorily initially without modifications. The test rig was useful for testing the performance of impacters after certain treatments or field trials (See **Part 2.4**). All impacters destined for field trials were tested exhaustively in the lab and checked they were within the design specifications before being sent out to the users.

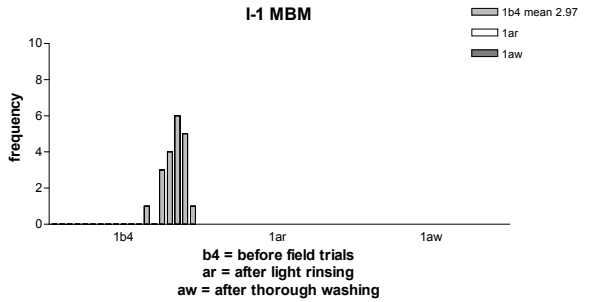
2.6 Evaluation of impacter performance in the field: An essential feature of the impacter design was to provide the desired impact force and to be able to deliver this impact repeatedly in the field over the growing season. During field use impacters are likely to be exposed to all sorts of conditions and potential mis-use so assessing how the design copes with these and to be able to detect if there is significant erosion of performance is very important. As part of the field evaluation of the impacter design detailed investigations were carried out on all of the Mk 4 prototype impacters before and after they were used for the 2004/5 field trials. Impacters were tested using the load cell test rig before they were delivered to the consortium field testers (b4), then immediately on receipt after completion of the field trials after several weeks use following a brief clean (ar) to remove accumulated dust and soil particles and then finally after more extensive cleaning and drying (aw). Impacters were evaluated for the reproducibility of the force delivered and the spread of forces delivered using the load cell test rig. The forces delivered by twenty impacts were recorded from each impacter for the three conditions described above – b4, ar and aw. The positive control for this experiment was a single impacter which was not used for the field trials and was evaluated along with those used for the field trials. Note that not all of the impacters were returned to Durham after the field trials – only eight sets of complete data were obtained. Firstly all the impacters performed acceptably before they were sent out to the field testers and all delivered the expected force. Secondly, upon return to Durham most of impacters were still performing within specification according to the mean force delivered and five showed almost no deterioration in performance. However it was clear that performance had deteriorated in the others to the extent that the spread of forces delivered was wider – i.e. the reproducibility had diminished. While this appeared to be a feature of several impacters it was particularly notable in two examples – I3 (ADAS) and I10 (Branston) and the latter gave an unacceptable performance with several impact forces below specification. Thirdly, the question then was could the reproducibility be recovered by a much more thorough cleaning. In all bar one of the impacters it was clearly the case that a thorough cleaning (washing in warm detergent), rinsing and drying process was capable of returning the impacters to their original specification. I10 appeared to be damaged to the extent that it still gave an unsatisfactory performance and would have failed quality control specifications. The reason for the deterioration in performance was most likely was due to soil particles accumulating in the moving parts of the impacter, particularly the piston and impact head. The differences in performance deterioration between testers most likely reflects different end-user treatment, cleaning and storage.

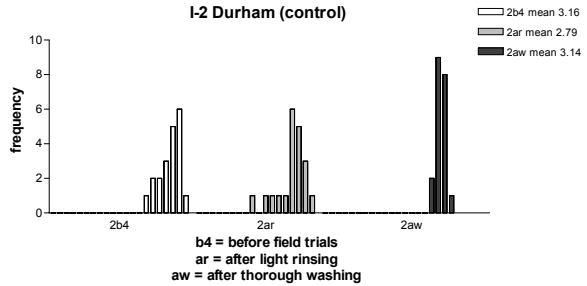
Conclusions: Field use of the impacters led to a deterioration of performance in the form of poorer reproducibility which could not be remedied by a superficial cleaning but required a more thorough cleaning to return the impacters to their original specification.
The longer term use of the impacters was not undertaken in this project but further deterioration might be expected through 'wear and tear' by soil particles abrading the aluminium surfaces of the moving parts.
Recommendations: Instructions for use should be modified to indicate that the impact head of the impacter should be wiped between impacts or set of impacts to minimize soil particles adhering and being drawn up inside the barrel. Where possible test tubers should be washed briefly to remove soil particles. Clearly this would not be possible for tests conducted actually 'in the field'. Instructions should also be included for the routine cleaning and maintenance of the impacters after each days testing. It may be necessary for the manufacturer to provide a regular quality control testing and servicing option to maintain impacter performance within the specification after each growing season. It would be advisable to conduct more extensive trials on manufactured impacters after field use with the modified 'instructions for use', to check that performance is maintained. Anodising of the aluminium surfaces might be a useful step to minimize such damage and reduce corrosion (See **Section 2.3**).

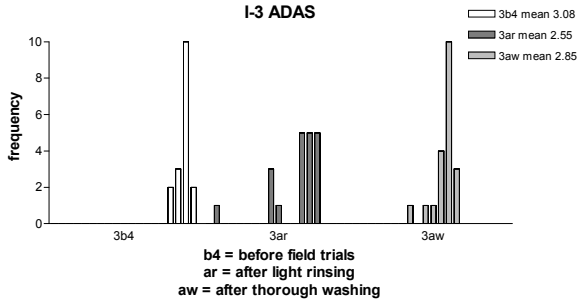
Example modified description for inclusion in the Instruction manual
" It is important for the continued functioning of the device that it is kept clean and free from dirt and soil particles – especially the shaft to the cocking handle, the inside of the barrel and the release button. Rinsing in warm water after use and storing in a dry area is ideal ".

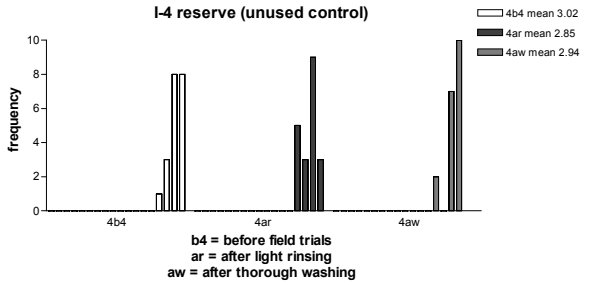
2.7 Note about end-user feedback on field use of impacters: Feedback and data on the performance of the various impacter designs was provided after the field trials at full consortium meetings or science sub-group meetings. These items will have been reported in the minutes of these meetings. Some feedback was communicated directly to Durham by the end-users. In all cases the comments, in relation to the criteria listed previously (**Part 2.2**) were fed back into the modified designs for the next generation of impacters. Examples of the end-user experience are provided in various experimental reports presented here (**Parts 2.10 and later sections)**

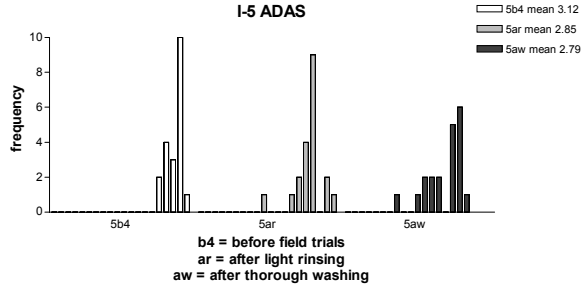
Figure 2.8 - Evaluation of Mk4 impacter performances after field use. Impact forces were measured by the force link load cell and charge meter (**Figure 2.7**). The forces delivered by 20 impacts were recorded for each of the eight impactors tested and plotted as frequency the measured force was achieved against force. Data are plotted in groups of three corresponding to before (b4), after rinsing (ar) and after thorough washing (aw). The mean forces for each set are indicated.

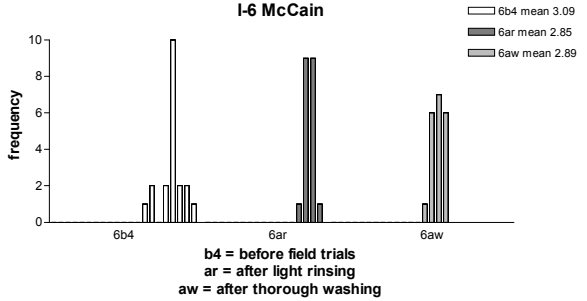


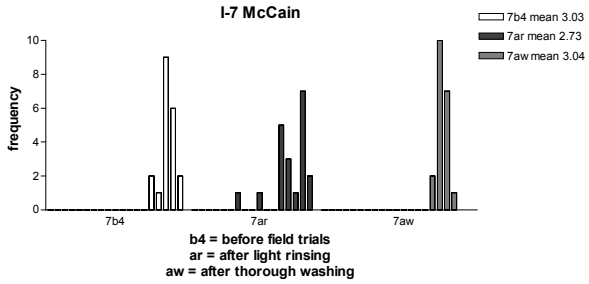


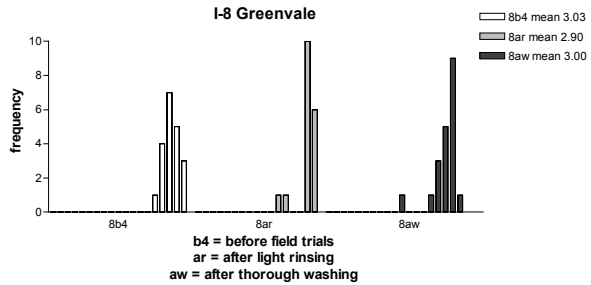


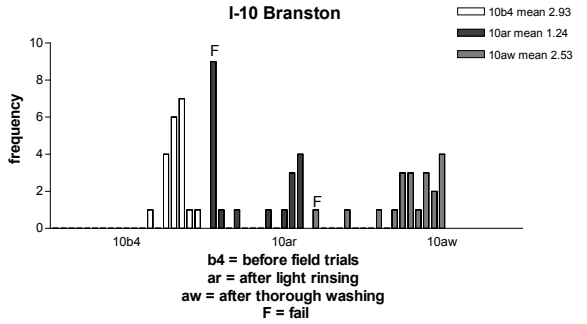






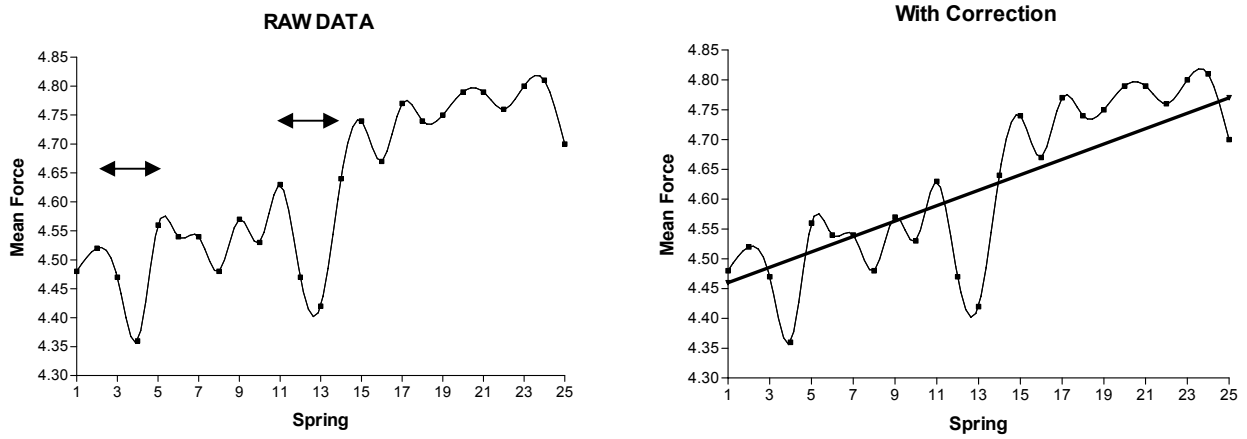






2.8 Note about temperature effects: It became apparent during extensive lab trials on the reproducibility of impacter force delivery with different samples of springs, that the force of impact changed measurably during the period of testing. The actual changes were relatively small but measurable using the link force load cell system (**Part 2.5**) but are somewhat exaggerated visually by the scales used in **Figure 2.9**. Experiments started early in the morning and involved fitting a test spring into one impacter – performing the test comprising of 20 impacts, recording each force and then calculating the mean. The spring was then replaced with a second test spring in the same impacter and the testing repeated. This was repeated throughout the day with 25 different test springs. The lab temperature during this time changed markedly from <15^{o}C to more than 20^{o}C. It was apparent that as the ambient temperature increased during the day the mean force of impact increased (**Figure 2.9**). Interestingly when the impacter was not used for short periods as indicated by the double arrows in **Figure 2.9** (tea and lunch breaks) the force of impact decreased again. After correcting for external temperature variation the springs perform almost identically (<0.5%). Estimates of the quantitative effect of the temperature rise indicates that a 4^{o}C increase in temperature caused a 6% increase in impact force. It is not clear how this might affect Bruise Index in the field.

Figure 2.9 – Temperature effects on impacter performance



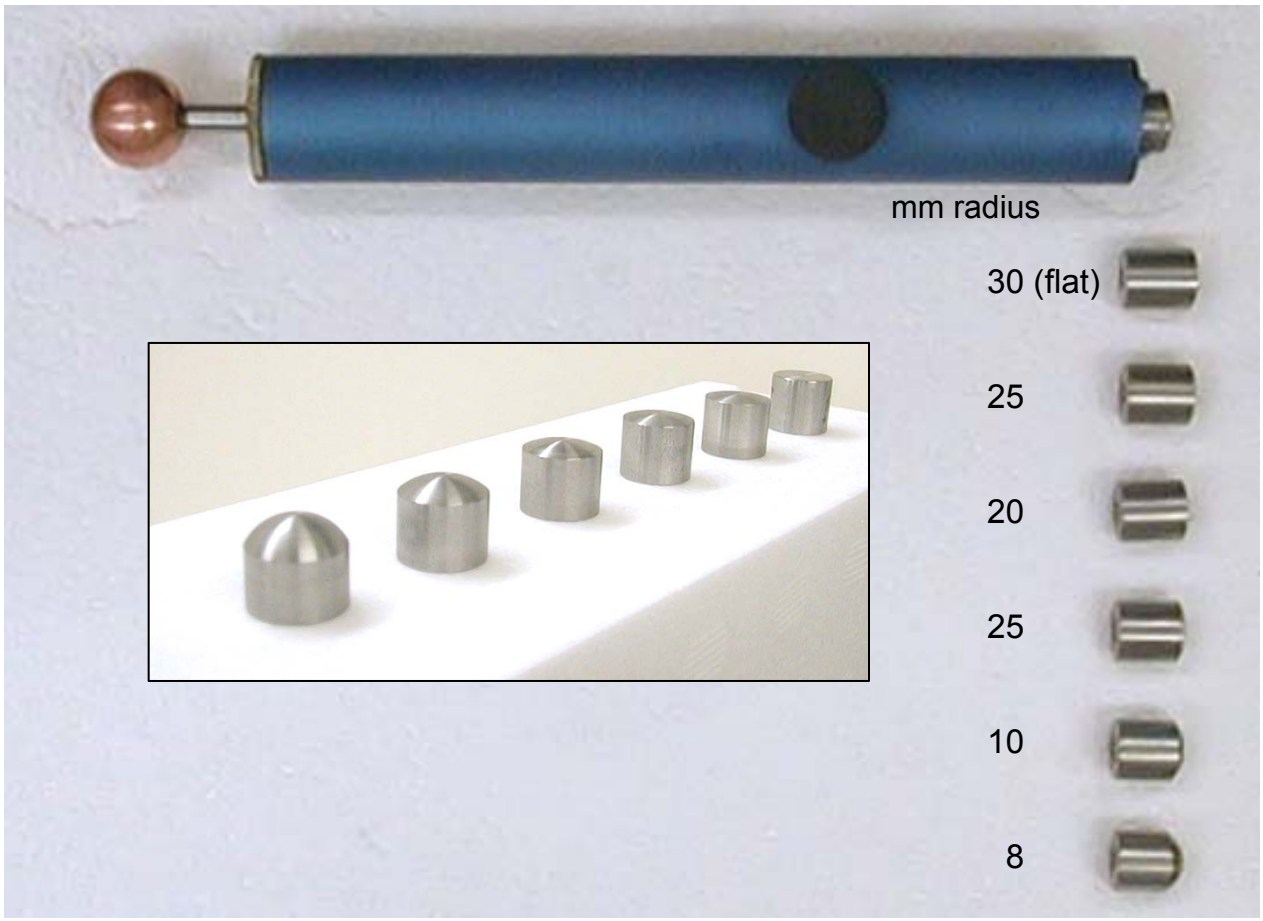
Conclusions: Appreciable temperature rises can affect the performance of the impacters leading to a variation in the force of impact. Within certain ranges this should not affect the validity of the radical assay but further work would be necessary to actually quantify how such a variation might alter predicted bruise indexes.

Recommendations: avoid exposing impacters to temperature rises – don't handle the barrel continuously; keep impacters out of direct sunlight and away from radiators or other sources of heat or cold. Allow the impacter to equilibrate with the ambient temperature.

2.9 Experiments to select the optimum impacter head shape (radius):

Stainless steel impacter heads were machined to provide a range of radii as indicated and to fit the shaft of one of the Mk 3 impacter prototypes.Tubers of a bruise susceptible variety (*cv* Russet Burbank) were used throughout these tests to provide a good level of bruising and radical generation. Sets of 20 tubers were impacted with each of the above impacter heads using the same impacter device. The impacter was evaluated using the load cell instrument before and after each experiment to check for delivery of a reproducible force. The impacted tubers were examined for a range of different parameters including: mean bruise intensity (mbi) – visual assessment of the intensity of pigment generation after 24h incubation; cracking – physical splitting of the tuber tissues in the damage zone; pitting – tuber surface compression; superoxide generation using the standard assay system on 5 of the impacted tubers; Durham bruise index, based on the dimensions of the bruise pigmentation zone and the bruise intensity; non-bruising tubers. The results were recorded and the data processed and plotted using Graphpad prism. The graphs are shown in **figure 2.12**.

Figure 2.10 – Radii used for impacter head shape evaluations. The flat head (30mm radius) was included for completeness of the dataset.

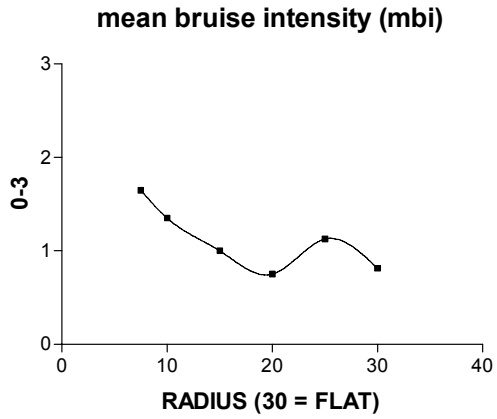


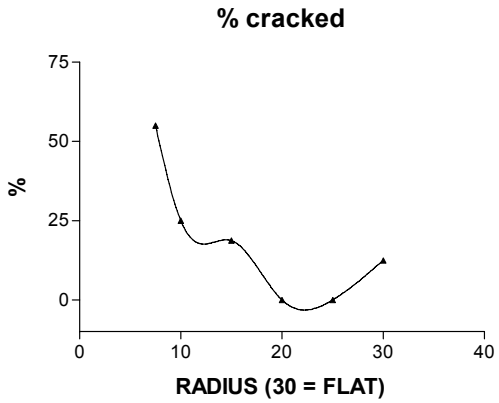
The conclusions from this evaluation are clear cut and interesting – essentially the sharper the head shape (smaller radii) the higher the level of damage caused as measured by any of the parameters used. This is an important consideration because even using only a moderate level of energy input, if this is 'concentrated' through too small an impact zone the damage required to induce blackspot bruising may be exceeded causing additional forms of damage such as surface pitting and sub dermal tissue cracking. With the superoxide assay the highest levels of generation were measured with the sharper heads although the increase between 20 down to 8 was only relatively small compared with the large increase in cracking and pitting damage over the same range of radii. Taking these latter results into consideration it was clear that a head radius of 15mm was optimal for bruise damage and superoxide generation while minimizing the other forms of physical damage. The flatter the head used the lower the damage incurred and the more variable the results become. The percentage non-bruised tubers also increases significantly with the flatter head shapes.

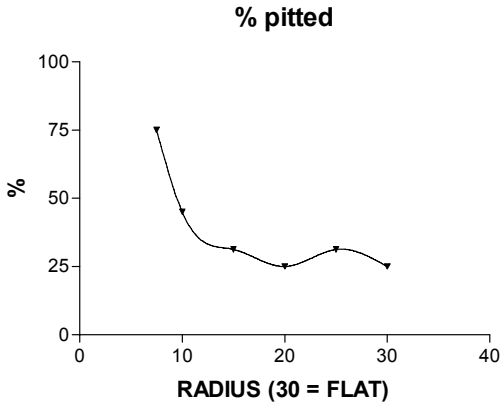
Conclusions: The results indicated that a radius of 15mm was suitable for delivering a force suitable for inducing an appropriate level of radical generation. This shape was also optimal for bruising using this impacter. Sharper shapes caused more cracking of the tissues while flatter shapes gave rise to increased variability, inadequate radical generation and poor bruising.

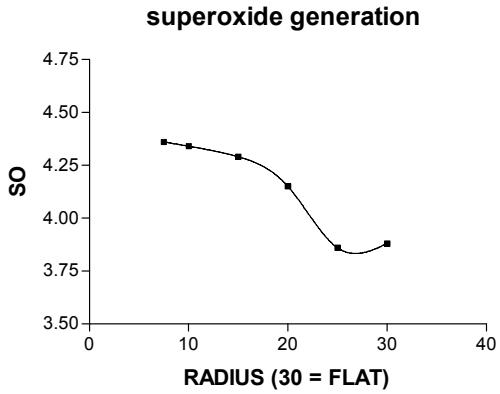
Recommendations: The shape of impacter head should be standardized on a 15mm radius for all future impacter designs. It is worth some consideration as to how impacter head shape relates to the force and energy of impact and how the underlying tissue volume is affected by this.

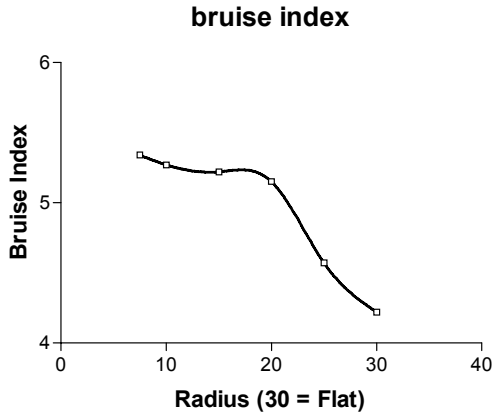
Figure 2.11 – Impacter head shape evaluations. Evaluation of the optimum impact head shape was assessed by several measured parameters as described in the text. In each case the increasing radius of the impacter head is plotted on the horizontal axis from sharpest (lhs) to flattest (rhs). Based primarily on the bruise index, superoxide generation and other damage the optimum radius was set at 15mm

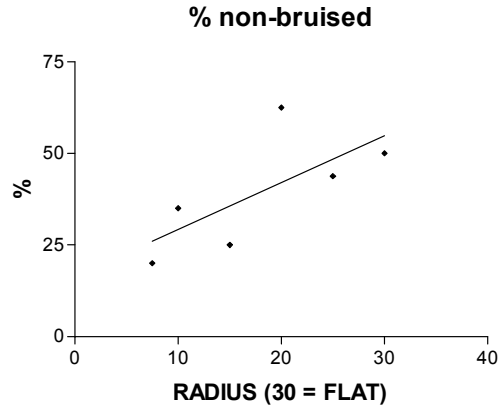










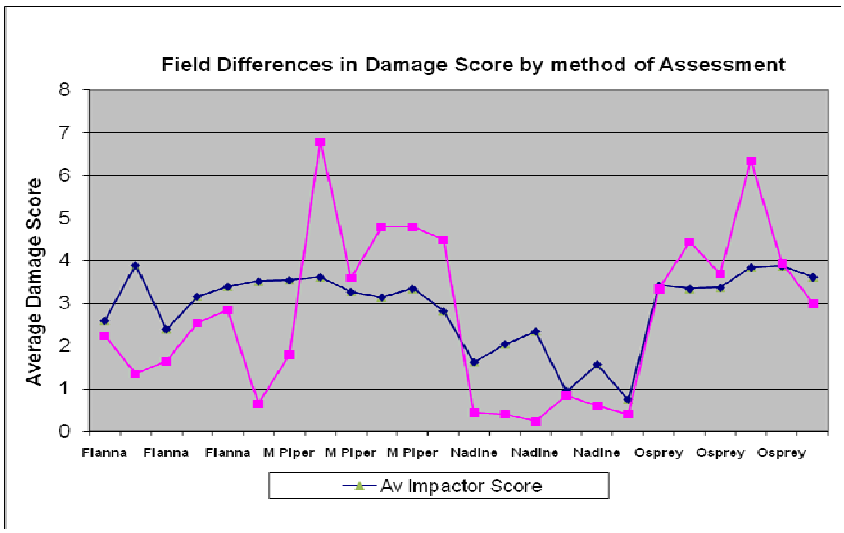


2.10 Comparison of impacter and bruise barrel for bruise assessment:

This work was carried out by D.Nelson and colleagues at Branston Potatoes Ltd. Standard bruise barrel bruise assays were carried out on a selection of cultivars grown by Branston (full experimental details not available). The same cultivars were also tested using the impacter to bruise the tubers. Damage scores revealed by each method were plotted against batches of tubers tested (**Figure 2.12a**). The average impacter damage scores and the average bruise barrel damage scores for the four cultivars tested were compared to the standard field harvest damage score for the same cultivars in **Figure 2.12b**).

Figure 2.12 - Comparison of Mk4 impacter performance with bruise barrel technique

a)



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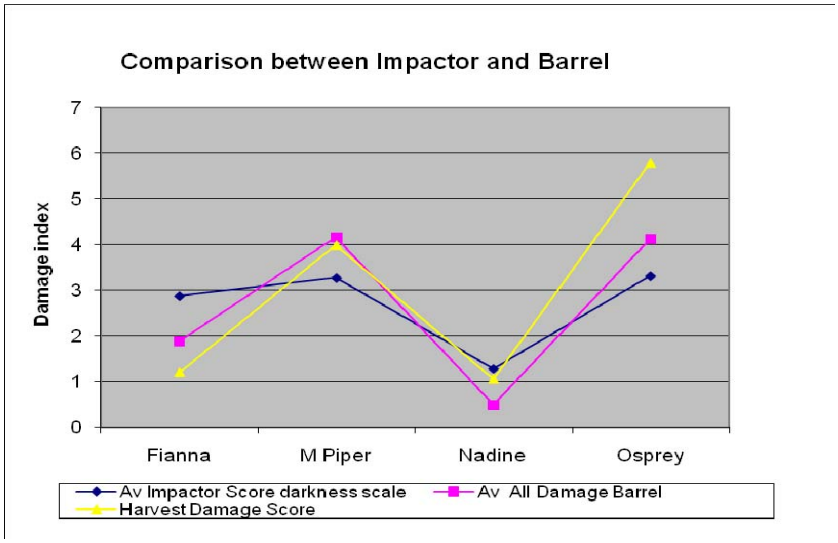


Conclusions: The results show a reasonable correlation between the bruise barrel method of assessment and bruising measured using the impacter. Generally the results using the bruise barrel were more variable showing a wider spread of scores than those obtained using the impacter. The trends were similar but the impacter appears to give results in a narrower range of scores – i.e. results for each cultivar were more closely similar. When the three mean damage scores, bruise barrel, impacter and harvest, were compared the trends were also very similar.

Recommendations: This makes the impacter a useful tool for the convenient estimation of bruise damage score and could be provided as a commercial tool for the industry in its own right. These correlation studies were essential to establish the validity for substituting a new test for an established industry standard test. Further correlative tests will be necessary to compare other methods of damage assessment employed by different sectors of the industry.

2.11 Final pre-production prototype impacter – Mk5 'Lishman Bruiser': Based on the Mk 4 cylindrical design and incorporating the issues raised by users during field trials with this impacter, the Mk 5 impacter was designed and constructed by Martin Lishman Ltd as the final pre-production prototype. The Mk 5 incorporated cylindrical barrel, upper trigger release, more ergonomic handle for easier priming and a modified tuber marking system (See **Part 2.13**). Apart from optimized performance and construction the design and finish was also selected to match the newly designed corer (See **Part 2.14**). As with the Mk 4 design the force delivered and the reproducibility of the Mk 5 design depended on high quality, uniform springs. Early in the production of these there was a problem with the supply of such springs and it was clear in the absence of a direct spring testing system that quality controls of the manufactured impacters would need to be stringent enough to eliminate those containing springs below the required specification. It would be a simple matter to replace the spring and re-test the modified impacter. At this point the full testing gear comprising of force link load cell, impacter clamp and charge meter were transferred to Martin Lishman Ltd. to set up the manufacturing quality controls. Sufficient Mk 5 impacters were manufactured for consortium members to perform further field tests.

Figure 2.13 - Design of the Mk 5 'Lishman Bruiser'. This was the final pre-production prototype and this model was most similar to the expected production model.

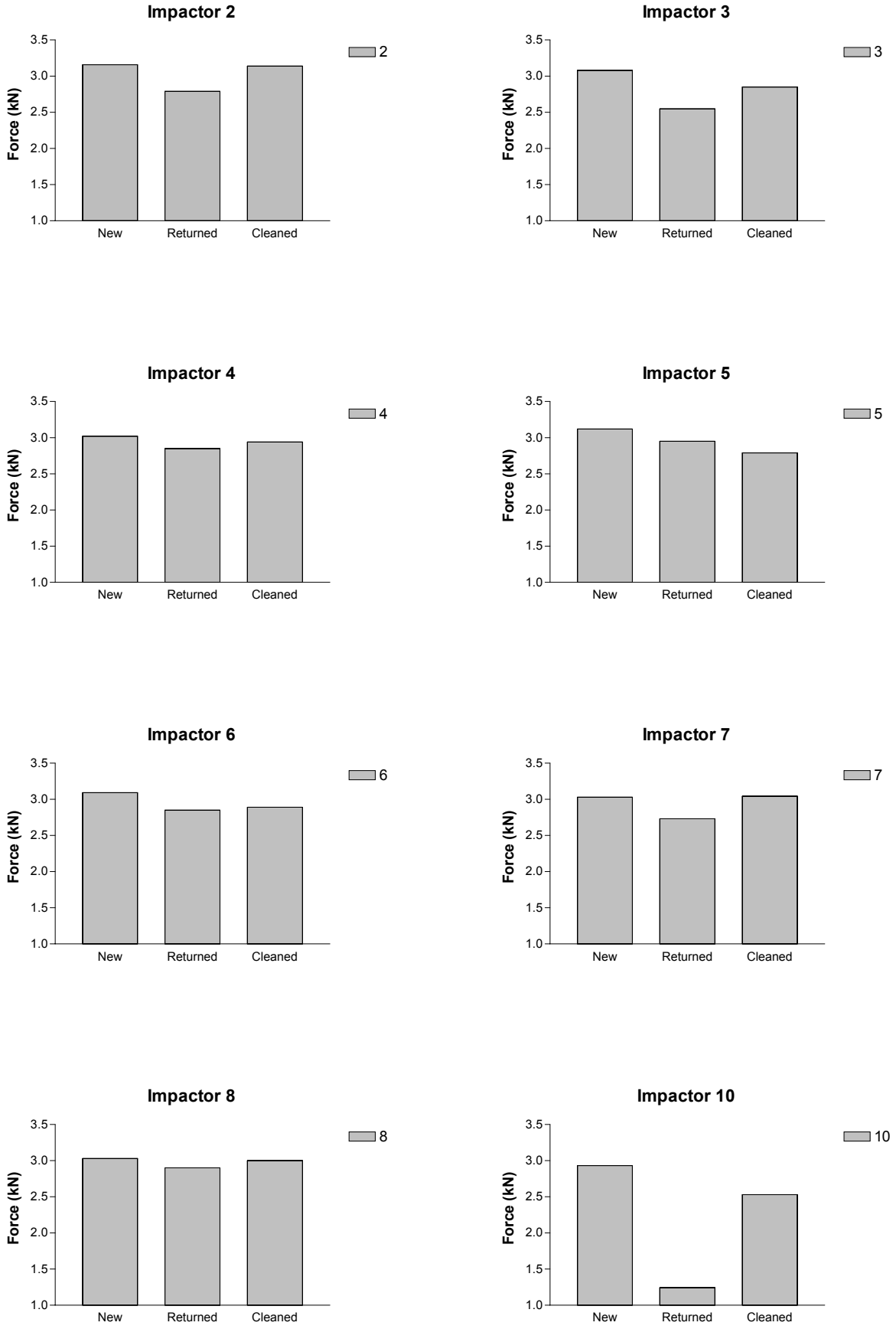
a)

b)

c)

2.12 Evaluation of Mk5 impacter performances after field use:

Figure 2.14 Evaluation of Mk5 impacter ('Lishman Bruiser') performances after field use. Results were very similar to those recorded using the returned Mk4 impacters after field use (**Part 2.6)**.

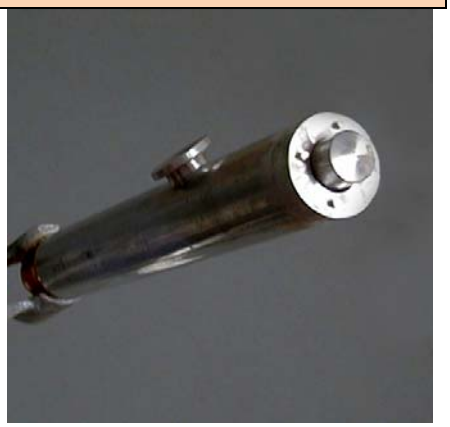


2.13 Design of the impacter marking device: The early impacters (Mks1 and 2) required the position of the damage zone to be marked immediately after the impact was delivered In most cases their was no surface blemishes to indicate exactly where contact between the impact head and the tuber surface had taken place. This was a particular problem with tubers with adhering soil or with a rough skin. Marking was usually done with a permanent marker pen in the centre of the impacted area. Clearly this was unsatisfactory and could lead to serious inaccuracies in the estimates of SO generation if the impact zone could not be clearly identified and excised with the coring device. Subsequent impacters (Mk3 – Mk5) were designed with small integral posts protruding from the end of the impacter barrel. In use the end of the barrel was pushed firmly against the surface of the tuber such that the posts created a pattern of holes in the tuber tissue flanking the impacted area. This was a distinct improvement providing a clear location of the point of impact and correctly positioning the barrel prior to impacting (**Figure 2.5**). These posts were however difficult and expensive to engineer into the impacter. The final Mk5 incorporated an integral circular cutting blade which proved superior to all previous marking devices. Effects on SO assays by these were minimal.

Figure 2.15 – Design of the impacter tuber marking device

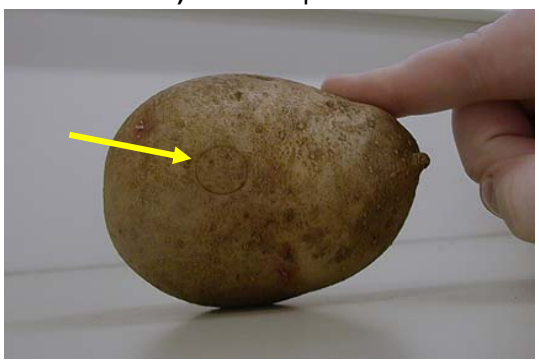
a) Mk 3 impacter

b) Mk 4 impacter



c) Mk 5 impacter

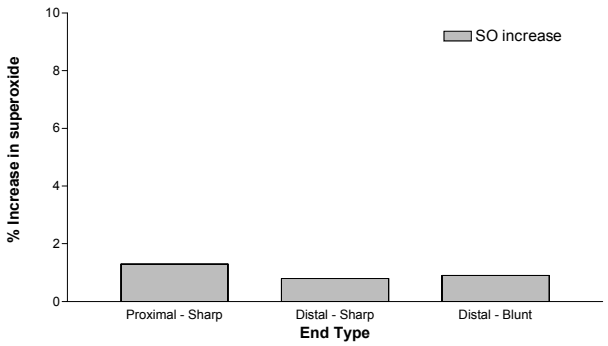
d) Alternative Mk5 ends employing integral cutting rings in the end of the barrels – proximal sharp, distal sharp and distal blunt



e) Efficient and clear marking of the impact zone with circular cutter design.

f) SO assays were carried out on non-impacted tubers which had been marked with the three different end designs to check for the mean contribution to SO levels caused by physical damage due to the cutters. The results indicated minimal damage and a negligible contribution to the overall impact-induced SO generation. The mean estimates indicated increases of between only 1% and 1.5%.

g)



Conclusions and recommendations: The damage caused by any of the designs for marking the surface of the tubers was minimal as judged by the SO assay and would not influence the impact induced SO generation significantly. The estimates of the increase in SO generation caused only by the process of marking the point of contact between the impacter barrel and the tuber surface was put at about 1.5% of the Thus the design which provided the clearest demarcation of the impact zone was chosen for the final impacter. This was the proximal sharp cutter which produced a circular indentation completely surrounding the impact zone (**Figure 2.15e)**).

2.14 Coring device design and evaluation: The original coring device (**Figure 2.2**) performed reasonably well in laboratory tests, excising reproducible half cores of a suitable size for the assay. However the half cores were found to be very difficult to remove from the device without further damaging the tissues – required a sharp ended device such as a needle or scalpel blade to remove the tissue from the corer. The subsequent coring devices were designed to incorporate a push-button plunger to facilitate ejection of the excised core halves. This facility allowed tissue samples to be taken and ejected directly into the assay bottles and XTT solution, minimizing possible contamination from surfaces or by handling the tissue pieces.

Figure 2.16 – Original design of corer (Mk 1)

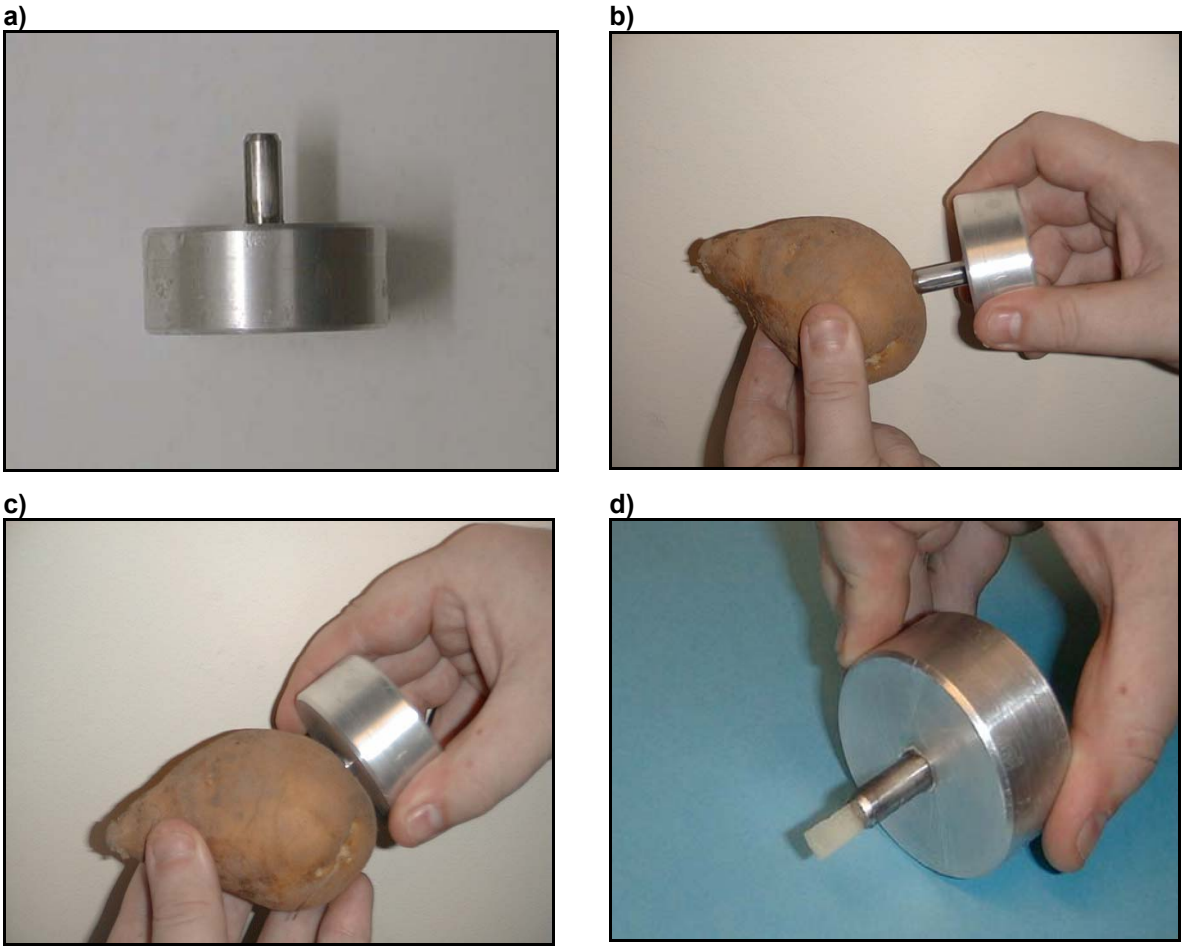
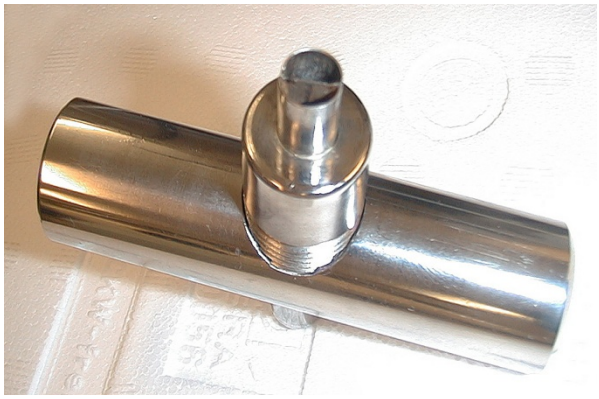


Figure 2.17 – Design of the 2nd (Mk 2) prototype coring device



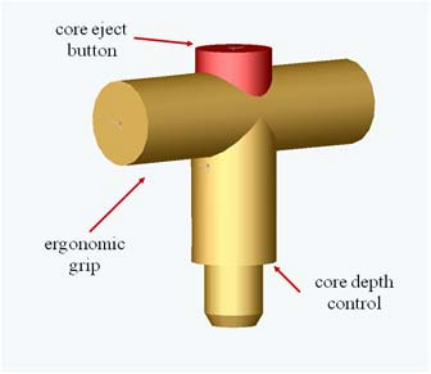
The Mk 2 corer prototype was constructed from solid stainless steel (**figure 2.3**). A total of three of these corers were constructed. While this device was extremely durable and efficient at producing reproducible tissue explants it was extremely heavy and could not be used for prolonged periods. The design of the push button plunger was excellent and worked very efficiently at ejecting the cut cores. The 'T' shaped design was also judged to be too uncomfortable for frequent use and some sort of asymmetric handle was thought could be better for handling and turning to cut the tissue. The blade which is inserted into the cutting tube to shear the tissue core allowing its extraction was difficult to insert and microweld into place. Also machining in stainless steel is difficult and expensive. These were perceived to be a problem for medium–scale manufacture. Suggested improvements to the design were the manufacture of new corer prototypes constructed from aluminium and plastic which would be much lighter and easier to machine; and to investigate alternative methods for fixing the cutting blade in place.

The Mk 3 corer (**Figure 2.18**) was highly successful and a big improvement on its forerunners. The design incorporated all the improvements following the lab and field evaluation of the Mk2 version. These included engineering the handle, barrel and cutting tube out of aluminium and using plastic parts for the plunger and the push button. The handle was offset to provide for more comfortable use when rotating the corer to excise the tissue pieces. Overall the corer was appreciably lighter and much easier to use over prolonged periods. The plastic plunger and push button worked better than the stainless steel version. The Mk 3 corers were anodized to protect them from scratching and corrosion. A total of ten of these corers were produced to provide all the consortium partners with one or more devices for testing. This version was used for the field evaluation of the impacters and the Blackspot Detect kit and also for many of the lab-based experiments. Both the Mk 2 and Mk 3 designs had the drawback of the ejector and push button sticking if the corer was not properly cleaned after use. In extreme cases the whole ejector mechanism seized up and could only be freed by a thorough soaking and washing. This is caused primarily by drying out of sugars and starch released from the tuber cut surfaces inside the cutting tube and plunger. This is an end-user maintenance problem and is unlikely to be solved by engineering alone.

Figure 2.19 shows the final and pre-production prototype of the coring device – the Mk 4 corer. This design moved away from the 'T' shape to a cylindrical shape in keeping with the Mk 5 impacter which was constructed at the same time. The reason for the change in design was partly for ease and economy of engineering, partly because it was felt that a cylindrical shape would be more ergonomic for rotating and partly for aesthetic reasons matching the appearance of the impacter. It was envisaged that the two devices would be sold as a boxed pair as shown in **Figure 2.19 b)**. The corer was engineered entirely from aluminium including the plunger and push button. Field testing of this corer showed it to be more susceptible to sticking and jamming possibly because of the aluminium construction. In some cases even soaking was unable to free the plunger mechanism. Despite investigation into alternative ways of fixing the cutting blade none was found to improve on the original system which was retained for the manufacture of the Mk3 corers. Enough Mk3 corers were produced to supply all the consortium partners.

Figure 2.18 – Design of the 3rd (Mk 3) prototype coring device. a) virtual corer designed using Autocad and incorporating the improvements suggested after lab and field use of the Mk2 corer. **b)** Mk3 corer along with the other items of hardware in a 'mock up' of the Blackspot Detect kit. **c)** the Mk3 corer engineered in aluminium (handle, barrel and cutting tube) and plastic (plunger and ejector button), showing the offset handle design and retained cutting blade. **d)** Mk3 corer in use cutting a tissue sample from an impacted tuber. **e)** End view of corer showing the excised tuber tissue prior to ejection from the cutting tube. **f)** Ejecting the two half cores from the corer into the assay bottle containing the XTT solution.

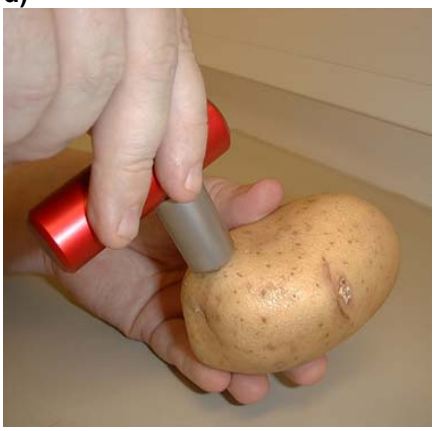
a)



b)

c)

d)



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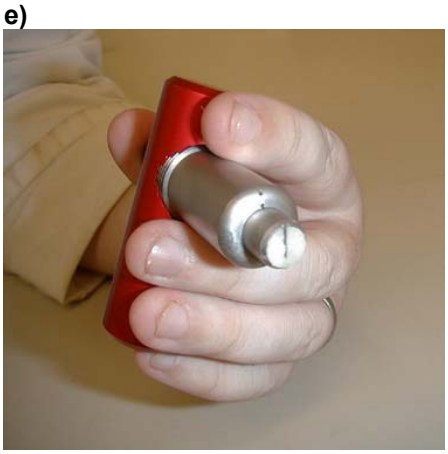


f)

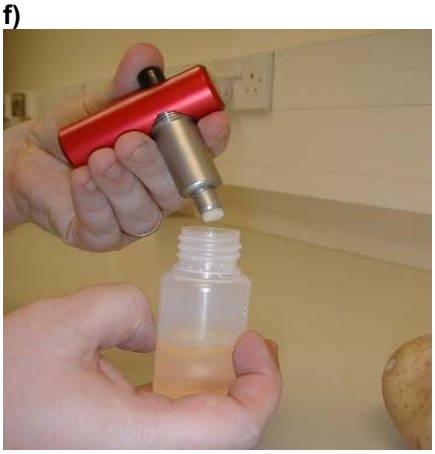


Figure 2.19 - Design of the 4th (Mk 4) pre-production coring device a) the Mk4 corer **b)** matching designs of Mk4 corer and Mk 5 impacter. Construction in a cylindrical format similar to the geometry of the impacter was felt to be easier to use for longer periods and also easier to manufacture.

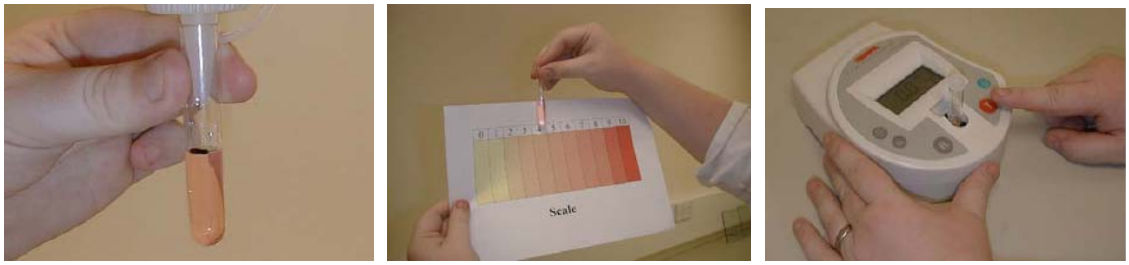
a)

b)

2.15 Colour scale charts and colorimeter evaluations: During the course of the LINK programme work on the colorimeter evaluations was carried out in Durham by Croy, Johnson and Hammond and at Martin Lishman Ltd by Gavin Lishman with feedback from the consortium partners who used the colorimeters for field trials. The original plan for the kit incorporated a visual chart of varying colour intensities corresponding to the amount of coloured formazan product produced in the assays. The changes to the radical assay system described in **Part 1.5** made the visual chart system significantly less accurate and would rely on users being able to accurately estimate small changes in colour intensity / shade by eye. This clearly would lead to inaccuracies and errors in the assay results and thus the visual chart system was abandoned in favour of an electronic system for measuring the intensity of the assay colour. In the lab experiments all readings were taken on a research grade spectrophotometer – a standard biochemistry instrument for taking absorbance measurements. However this is a highly specialized piece of equipment costing many thousands of pounds so would be inappropriate for use in a kit where economics plays a major role in its success. Colorimeters are relatively cheap versions of the spectrophotometer though somewhat less accurate. Some evaluation of these instruments was essential to prove their utility for use in the kits.

Figure 2.20 – Example colour scale charts for visual estimation of bruise susceptibility

a) The original visual chart system with shades of blue based on the anticipated alkalinisation of the formazan product to produce a bright blue assay result. Subsequently this was shown to be an unstable system and was discontinued.



Bruise index	0	1	2	3	4	5	6	7	8	9	10

b) Visual chart developed for use with the XTT assay without alkalinisation (see photos above). In practice the assay results fell mainly into the range of 1-5 rather than the full range – the extreme end of the scale was only achievable by extending the 20 minute assay with a very susceptible variety or by chemical means.

Bruise index range	0 - 2	3 - 5	6 - 8	9 - 10

c) attempts to improve the usability of the chart system by 'pooling' the individual values producing fewer bruise index values but with a greater difference between them but this also proved to be unsatisfactory.

Conclusions: visual chart systems were judged too subjective in that the shade differential was too slight for accurate determination of any point on the scale. Also the variation in colour scale between colour printers and PC screens would make this method potentially very inaccurate and difficult to discriminate between bruise susceptibility classes. Using a digital camera to photograph standard assay results would suffer from the same problems of comparability and transferring colour scales to a portable medium.

Recommendations: Use more accurate electronic systems for estimating the colour intensity of the assay solutions and calibrate this for use to measure bruise index directly.

Evaluation of the colorimeters and comparison of performance with the research spectrophotometer instrument was carried out using a dilution series of a mixture of red and yellow food dyes which gave a good approximation to the XTT formazan colour intensities. The concentration was selected on the basis of the maximum intensity which gave a reading in the colorimeters (too high a concentration gives an out of range or 'flashing' readout) and then a dilution series prepared from this solution. (Note that the range of absorbances that the colorimeters were capable of reading was significantly less than the spectrophotometer which provides a linear readout of up to 3 or more OD units). Significantly this means that the colorimeters are more sensitive at the lower absorbance range as produced in the SO assays. The food dye dilutiuon series provided a suitable range of readings across all three instruments. Unlike the spectrophotometer which measures a precise path length of 1cm (the thickness of solution measured) the colorimeters could take tubes and cuvettes of different thickness up to 14mm. This provided some opportunity for improved sensitivity since more of the assay solution could be measured in the colorimeters. The WPA instrument (**Figure 2.21a**) was the more expensive colorimeter and gave a readout in absorbance units equivalent to that of the spectrophotometer, while the Hanna colorimeter (**Figure 2.21b**), being an instrument used primarily for water quality testing gave readings in water colour units. These units were proportional to absorbance but required to be converted from water colour units to absorbance, then corrected for differences in path length then conversion to bruise index values. The details of how this was carried out is provided in **Parts 2.17, 2.18, 2.19** below.

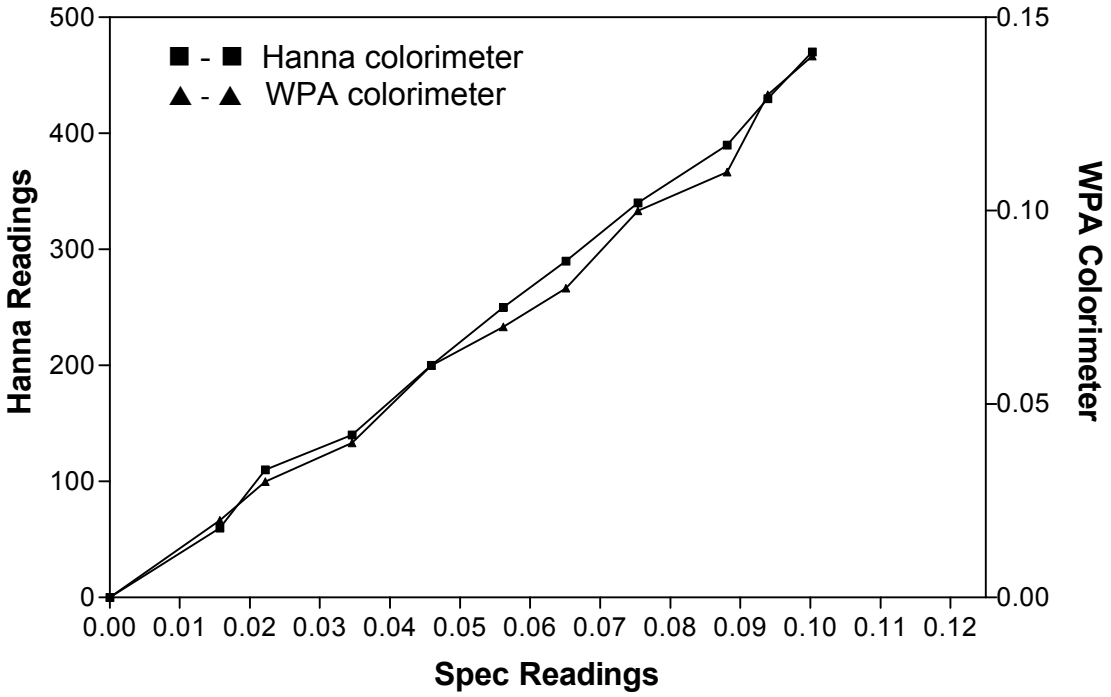
Immediately noteworthy from the direct comparison of measurements of standard solutions was the fact that both the colorimeters gave completely linear readings over the range of solutions tested (**Figure 2.22**). This indicates that these instruments were accurate and sensitive enough to be used for the kit application. A comparison of the actual values of the readouts from the colorimeters versus the spectrophotometer shows firstly that the colorimeters give almost identical readings and are essentially equivalent devices and secondly that the colorimeters give higher readings than the spectrophotometer indicating that they are more sensitive. This is somewhat erroneous since the spectrophotometer is accurate to at least 3 decimal places of an absorbance unit. The reason for the higher colorimeter readings is due to the longer path length in the colorimeters where tubes or cuvettes of 14mm diameter were used for the measurements. This provides a useful increase in sensitivity for the assay. At the time of the Hanna colorimeter evaluation a 20mm cuvette was supplied which would have provided even greater sensitivity. However this was too large to be accommodated in the HI93727 instrument supplied and although a larger path length instrument was being developed by Hanna no further information was available as to when this could be supplied.

Figure 2.21 – Colorimeters used in the kit development. a) *WPA Colourwave* CO7500 colorimeter **b)** *Hanna Instruments* HI93727 colorimeter. Readings on both instruments were compared with those taken on a Beckman diode array research spectrophotometer. The readings were plotted against the spectrophotometer readings in **Figure 2.22** below.

a) 

b) 

Figure 2.22 – Equivalence of readings and straight line relationship between colorimeters and spectrophotometer readings



2.16 Notes on feedback from field trials with the colorimeters: Several WPA instruments were purchased by the partners or were lent out for the field trials of the kit. No specific tubes or cuvettes were supplied with these instruments and no specific type or product was recommended. There were several reports back from the end users indicating problems with the use of the colorimeters (See also reports from the individual partners in **Part 3.11**). Most notable was a problem with variability in consecutive readings of the same assay samples. The kit sent out to the partners provided disposable tubes for measuring the assay colour intensity. These were of clear plastic (polystyrene) construction and relatively cheap. The philosophy in using these was they would be cheap to provide and would be safe and disposable - end-users would not need to wash them out for re-use. In practice the tubes were not absolutely optically correct and the surfaces were very fragile and subject to damage. The problem was discovered when the partners attempted to re-read the same solution several times consecutively – this was not a recommended procedure but was instigated by the end-users to ensure that the first reading was correct. The experiment indicated that the readings varied in some cases quite appreciably, when they should have been identical. On investigation it became clear that the variability was arising primarily for two reasons – firstly the tubes were not designed for accurate absorbance readings although the initial tests showed them to be satisfactory at the level of sensitivity required. Examination of the tubes showed that some of them had irregularities in the plastic so that depending on the position the tube was placed in the colorimeter the readings of any solution could vary slightly. Using exactly the same position in the instrument i.e. the tube is always placed in exactly the same orientation, alleviated the problem. Placing a marked line on the side of each tube would help orientate the tubes each time. Repeatedly reading the same tube produced scratches on the surface of the tubes caused by the tube gripping mechanism inside the colorimeter and contributed to the variation in readings – again orientating the tubes in exactly the same way each time the tube was read would alleviate this problem. Another problem encountered was the cleanliness of the outside of the tube. Any adhering debris, dust or dirt will cause erroneous readings due to scattering of the light in the colorimeter. Cleanliness in handling the instrument and assay tubes is of paramount importance for accurate, reproducible readings. This should be made clear in the instruction manual. The last problem reported was potentially the most serious in that the end-users reported that it was possible to insert the sample tube so that it was not vertical in the colorimeter but at a slight angle. This led to significant deviations in the readings. The problem was traced again to the sample tubes supplied – although these fitted into the sample chamber snugly it was possible that if inadequate care and attention was paid they could be incorrectly located. The only solution to this problem apart from dealing with it by appropriate end-user training was to select better fitting tubes and to provide ones which were of a better optical quality. Glass tubes were provided subsequently which helped reduce some of the variability but these could not be used in a disposable mode and had to be

washed out and recycled. Subsequently dedicated cuvettes as supplied with the Hanna colorimeters, solved most of the problems since these are optically correct containers and are located only in one specific position in the colorimeter. The cleanliness of the surfaces applies equally to any tubes or cuvettes used for sample measurements – this can only be addressed through the instruction manual and by end-user training.

Conclusions: problems with inaccuracies and variability in assay readings were primarily caused by inappropriate use and handling of plastic sample tubes. Problems were solved by modifying the instructions (more precise handling of tubes) or by replacing the plastic tubes with glass ones. The requirement for cleanliness when doing the assay was regarded as a serious drawback.

Recommendation: Change over to the use of dedicated cuvettes for the colorimeter instrument selected for supply with the kits. Ensure that appropriate instructions are provided in the instruction manual and

2.17 Specification for Conversion of Hanna 93727 Colour of Water ISM Instrument:

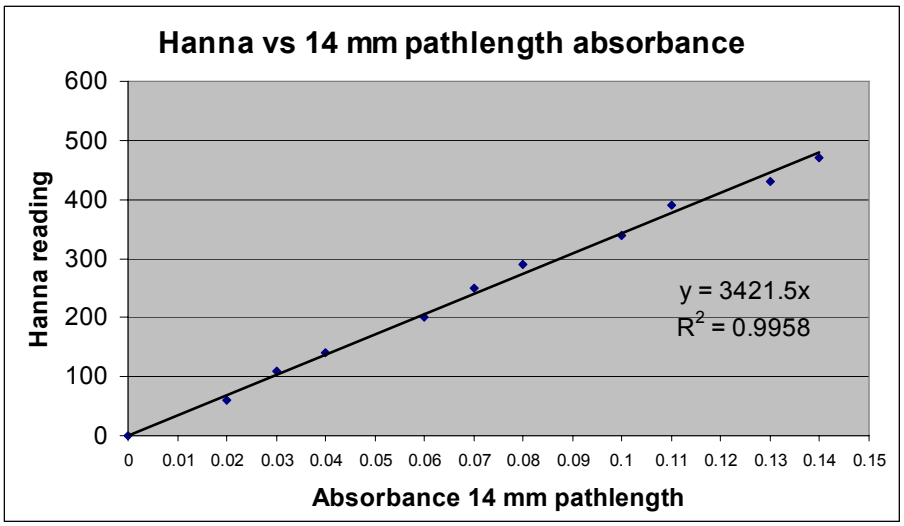
This section was provided by Tim Hammond and Ron Croy. The information contained within this part of the report relates also to **Part 4 Commercialisation**. **Parts 2.17, 2.18** and **2.19** are contained within **Appendices 6,7** and **8** supplied on the accompanying CD.

A dilution series was made on a red food dye to provide samples for measurement. Absorbances have been measured on a laboratory spectrometer (14mm path length) and this data correlated with the Hanna (15mm path length) colour of water units output. See **Figure 2.23** and **Table 2.1** below.

Table 2.1 Conversion between absorbance and Hanna output

Hanna Units 15 mm pathlength	Absorbance 14 mm pathlength
0	0
60	0.02
110	0.03
140	0.04
200	0.06
250	0.07
290	0.08
340	0.1
390	0.11
430	0.13
470	0.14

Figure 2.23 Conversion of standard absorbance data to Hanna water colour units



Conversion from the standard absorbance data into Hanna units can therefore be represented by the equation

Hanna output (**H**)= 3421.5 x Absorbance 14 mm path length (**A**)
or **H** = 3421.5 x **A**

Table 2.2 Summarises the relationship between bruise Index **B** and Absorbance (measured for 14mm path length **A**

Note that the Hanna Instrument is operating at a maximum sensitivity of 500 bruise units , equivalent to an absorbance of around 0.15 (measured on 14 mm path length). The sensitivity of the instrument will need to be reduced so that absorbances of **up to 0.65** can be accommodated

Table 2.2 Bruise index scale

A **Absorbance** 14 mm pathlength	B **Bruise Index**	H **Hanna** Calculated response
0	0	0
0.01	0.1	34.215
0.02	0.3	68.43
0.03	0.4	102.645
0.04	0.6	136.86
0.05	0.7	171.075
0.06	0.9	205.29
0.07	1.1	239.505
0.08	1.2	273.72
0.09	1.4	307.935
0.1	1.5	342.15
0.11	1.7	376.365
0.12	1.8	410.58
0.13	2	444.795
0.14	2.1	479.01
0.15	2.3	513.225
0.16	2.5	547.44
0.17	2.6	581.655
0.18	2.8	615.87
0.19	2.9	650.085
0.2	3.1	684.3
0.21	3.2	718.515
0.22	3.4	752.73
0.23	3.5	786.945
0.24	3.7	821.16
0.25	3.9	855.375
0.26	4	889.59
0.27	4.2	923.805
0.28	4.3	958.02
0.29	4.5	992.235
0.3	4.6	1026.45
0.31	4.8	1060.665
0.32	4.9	1094.88
0.33	5.1	1129.095
0.34	5.3	1163.31
0.35	5.4	1197.525
0.36	5.6	1231.74
0.37	5.7	1265.955
0.38	5.9	1300.17
0.39	6	1334.385
0.4	6.2	1368.6
0.41	6.3	1402.815
0.42	6.5	1437.03
0.43	6.6	1471.245
0.44	6.8	1505.46
0.45	7	1539.675
0.46	7.1	1573.89
0.47	7.3	1608.105
0.48	7.4	1642.32
0.49	7.6	1676.535
0.5	7.7	1710.75
0.51	7.9	1744.965
0.52	8	1779.18
0.53	8.2	1813.395
0.54	8.4	1847.61
0.55	8.5	1881.825
0.56	8.7	1916.04
0.57	8.8	1950.255
0.58	9	1984.47
0.59	9.1	2018.685
0.6	9.3	2052.9
0.61	9.4	2087.115
0.62	9.6	2121.33
0.63	9.8	2155.545
0.64	10	2189.76

The Hanna “Colour of Water” units response have been calculated using the equation derived from the fit to figure 1 and the results summarised below in figure 2.24

Figure 2.24 Bruise index conversion from Hanna display

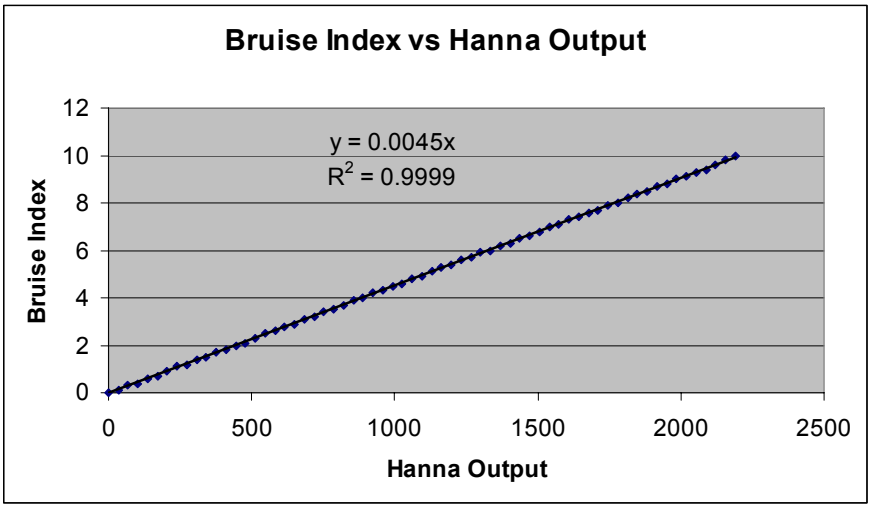


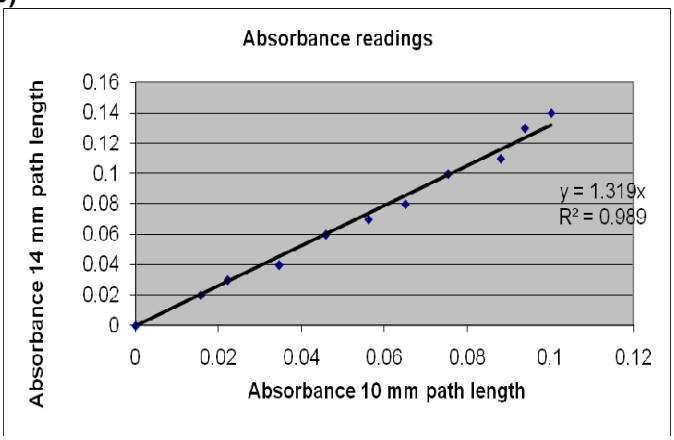
Figure 2.25 - Conversion of Hanna colorimeter scale to bruise index scale - summary

a)

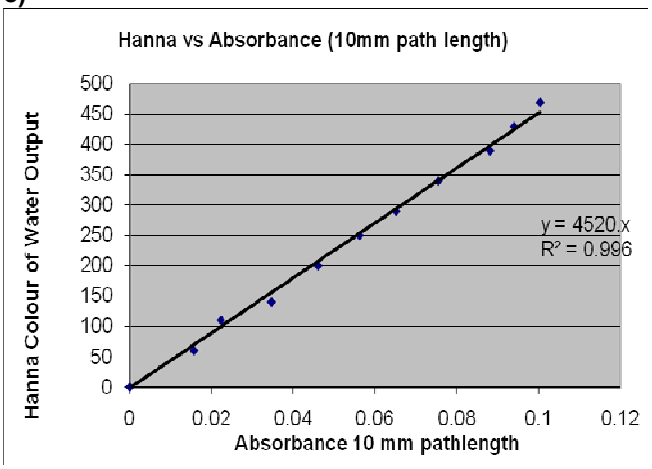
Colorimeter Read-off Chart

Colorimeter Reading	Bruise Index	Status	Colorimeter Reading	Bruise Index	Status
0.01	0.1	V.RESISTANT	0.33	5.1	MODERATE
0.02	0.3		0.34	5.3	
0.03	0.4		0.35	5.4	
0.04	0.6		0.36	5.6	SUSCEPTIBLE
0.05	0.7		0.37	5.7	
0.06	0.9		0.38	5.9	
0.07	1.1		0.39	6	
0.08	1.2		0.40	6.2	
0.09	1.4		0.41	6.3	
0.10	1.5		0.42	6.5	
0.11	1.7		0.43	6.6	
0.12	1.8		0.44	6.8	
0.13	2		0.45	7	
0.14	2.1		0.46	7.1	
0.15	2.3		0.47	7.3	
0.16	2.5		0.48	7.4	
0.17	2.6	RESISTANT	0.49	7.6	V.SUSCEPTIBLE
0.18	2.8		0.50	7.7	
0.19	2.9		0.51	7.9	
0.20	3.1		0.52	8	
0.21	3.2		0.53	8.2	
0.22	3.4		0.54	8.4	
0.23	3.5		0.55	8.5	
0.24	3.7		0.56	8.7	
0.25	3.9		0.57	8.8	
0.26	4		0.58	9	
0.27	4.2		0.59	9.1	
0.28	4.3		0.60	9.3	
0.29	4.5		0.61	9.4	
0.30	4.6	MODERATE	0.62	9.6	
0.31	4.8		0.63	9.8	
0.32	4.9		0.64	10	

b)



c)



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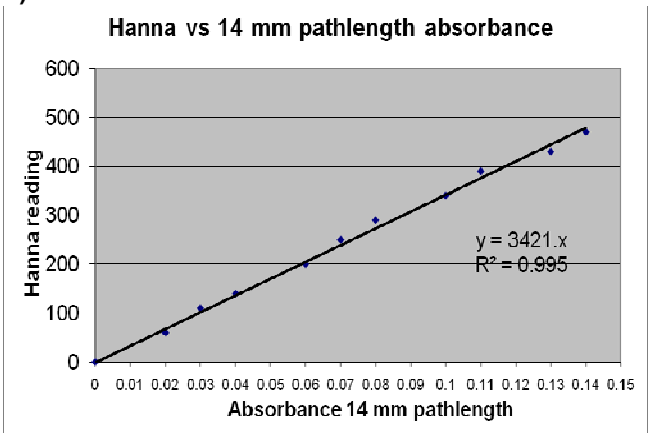

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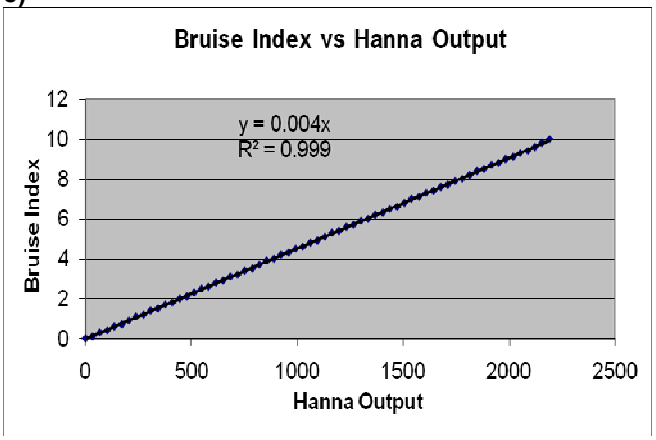


2.18 Assessment of scale conversion for Hanna test instrument to read bruise index units:

Appendix C3 defines the required modifications to the Hanna 93727 "colour of water" instrument to permit scale readouts in bruise index units.

Two Hanna units with reprogrammed EPROMS have been received from Hanna for assessment in the Durham laboratories.

Tests were run to compare the new instruments with the absorbance data obtained previously with a laboratory spectrometer to define bruise indices.

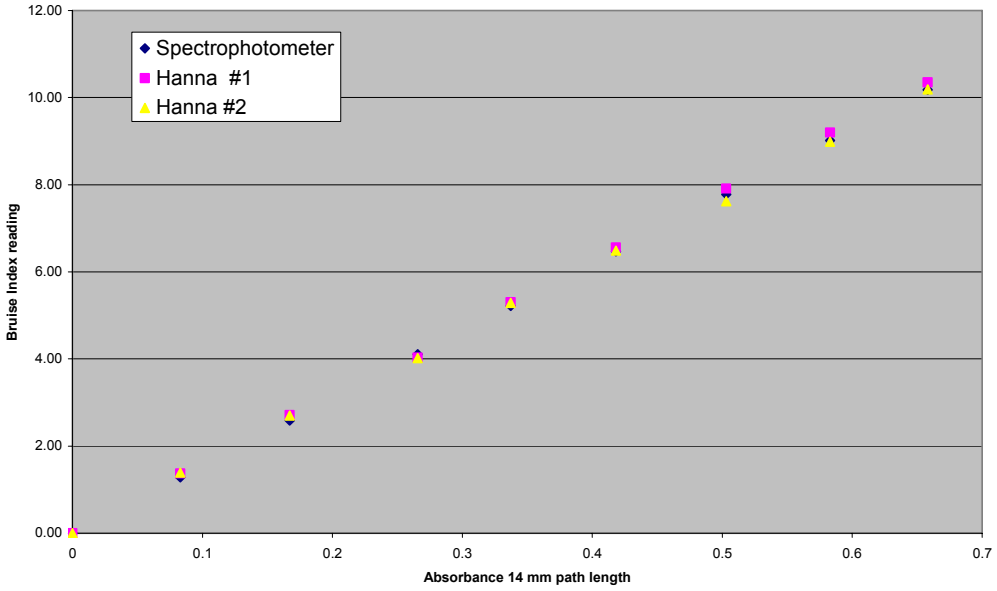
A red dye solution was used at progressive dilutions to provide a range of solutions with absorbances in the range required for bruise index measurement.

The laboratory spectrometer absorbances were converted to bruise indices using the conversion table given in Appendix 1 Table A2. These were compared with the direct bruise index readings from the two Hanna Instruments across the bruise index range 0 – 10 as defined below in Table 1 and Figure 1.

Table 2.3 Comparison of Hanna direct bruise index readings vs laboratory spectrometer

Spectrometer reading		Hanna Instr. #1	Hanna Instr. #2	
Absorbance	Bruise index	Bruise Index	Bruise Index	
0	0.00	0	0	
0.08288	1.28	1.37	1.39	
0.16716	2.59	2.71	2.7	
0.26558	4.11	4.03	4.02	
0.33712	5.21	5.3	5.29	
0.41818	6.47	6.56	6.49	
0.50302	7.78	7.91	7.62	
0.58296	9.02	9.2	8.99	
0.658	10.18	10.35	10.19	FLASHING

Figure 2.26 Plot of Hanna direct bruise index readings vs research spectrometer



An excellent correlation was achieved between the 3 instruments.

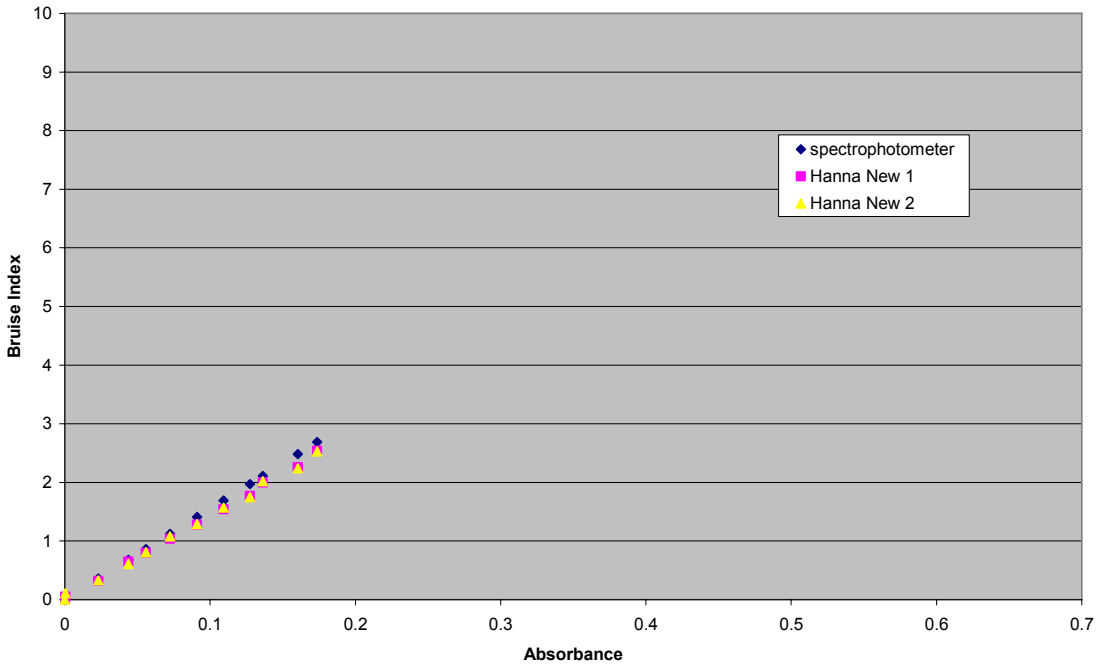
A second set of experiments were made at very low dye concentrations to confirm that the instruments yielded accurate bruise indices at the low absorbance end of the scale.

The data are summarised in Table 2 and figure 2. Again very good correlation was observed.

Table 2.4 Low concentration comparison of Hanna direct bruise index readings vs research spectrometer

Spectrometer reading Absorbance	Bruise index	Hanna Instr. #1 Bruise Index	Hanna Instr. #2 Bruise Index
0	0	0	0
0.00042	0.006497	0.05	0.11
0.0231	0.357311	0.32	0.33
0.04382	0.677808	0.65	0.61
0.05586	0.864042	0.8	0.81
0.07252	1.121739	1.04	1.08
0.09114	1.409754	1.28	1.29
0.1092	1.689106	1.54	1.57
0.1274	1.970623	1.77	1.75
0.13636	2.109216	2	2.02
0.16044	2.481686	2.26	2.24
0.17374	2.68741	2.54	2.53

Figure 2.27 Low concentration plot of Hanna direct bruise index readings vs laboratory spectrometer



Conclusion: The Hanna Instrument conversion from its original native water colour units to bruise index output has been successful with excellent correlation with the laboratory spectrometer data reported in previous years.

Recommendation: The Hanna colorimeter needs to be modified for bruise index readout according to the correlations described here and a modified colorimeter specific for use in the blackspot detect kit produced.

Part 3 - Studies on the development of field sampling methods and plant variability.

Objective 3 – Field Sampling Methods

- Task 11 – plant variation
- Task 12 – industrial trials and materials
- Task 13 – ADAS trials field variation / data handling

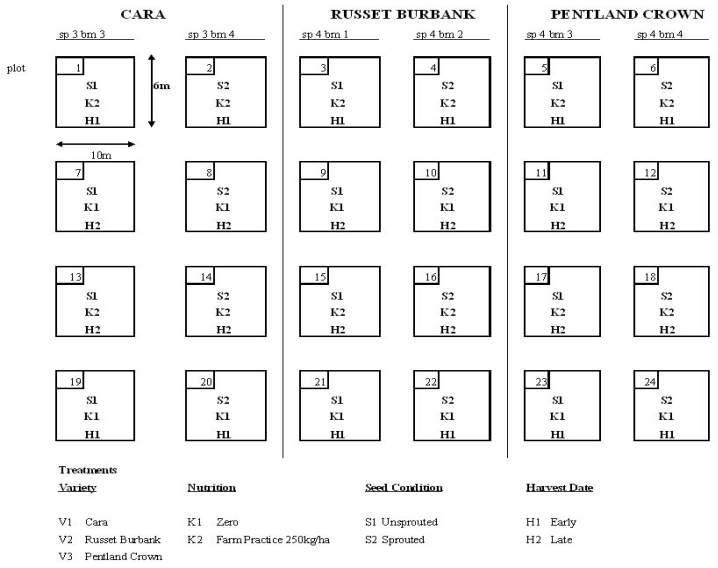
3.1 Results from studies of small-scale variability in bruise susceptibility (within & between plants).

Background: This work was performed jointly by Durham and ADAS in the 2003 and 2004 seasons. ADAS planned the field plots and grew the crops at ADAS Gleadthorpe and the hand lifting of the bulk of the crops. During 2004 for task 11 Durham harvested samples of the tubers and performed the detailed analyses of SO generation.

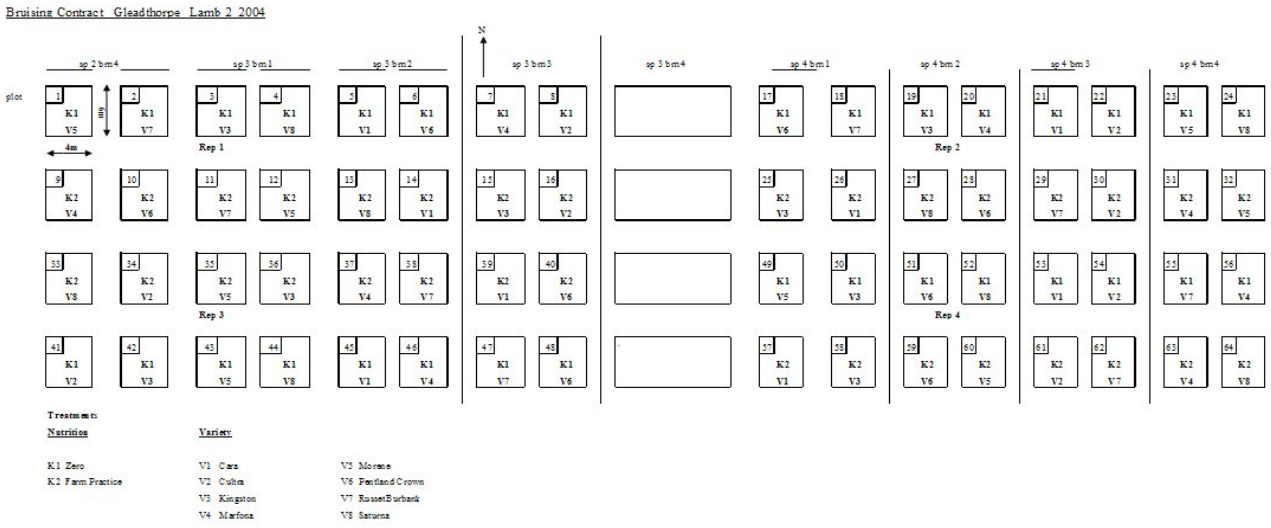
Figure 3.1 - Example field plots grown at ADAS Gleadthorpe. a) 2003 season, b) 2004 season. In addition to growing replicate plots of 8 varieties displaying different genetic pre-disposition to bruise susceptibility half the plots were exposed to low potassium (zero) nutrition to further test the hypothesis that low potash encouraged bruise susceptibility.

To achieve the best chance of producing crops displaying a range of bruise susceptibilities the following crops were grown in the 2003 season (**Figure 3.1a**).. Three varieties (*Cara* – resistant; *Pentland Crown* – moderate to resistant and *Russet Burbank* – Susceptible to highly susceptible) were grown under two potash regimes from two seed tuber conditions (unsprouted and sprouted) and samples were harvested at two dates (early and late). The layout of the field plots at Gleadthorpe is shown opposite. Based on the results of these trials in the following year, 2004, **seed condition** and **harvest date** were dropped as variables but different **potash nutrition** regimes were kept with eight varieties including *cv Cara, Cultra, Kingston, Marfona, Morene, Pentland Crown, Russet Burbank* and *Saturna.* (**Figure 3.1b**).

a)



b)



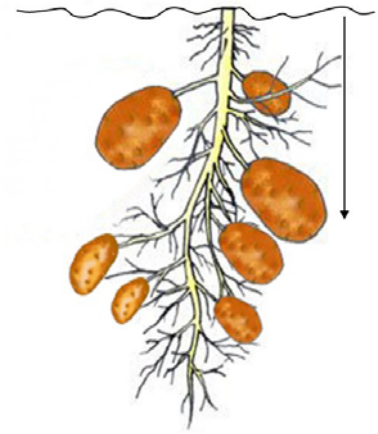
The procedure for field testing potato crops requires growers to select individual tubers at random from a minimum number of plants depending on the size of the field (see relevant parts of the ADAS report: **Part 3.3 - Formulation of a sampling strategy for commercial assessment of bruising susceptibility, Part 3.4 - Field work and data analysis, 2005 and Part 3.5 - Variation in potato bruising and implications for sampling**). An important objective was to ascertain if any tuber within an individual plant, would be representative of all the tubers from that plant in its bruise/SO generation response to impact and would this tuber also be representative of the other parts of the field. No data were available from any sources on the spatial variation within individual plants either depthwise or with the radius from the central stem so these experiments were unique and only possible using the technology developed in this programme. A detailed experiment was set up to test the bruise susceptibility of individually sampled and located tubers selected from random field grown plots.

Materials and Methods: Eight potato cultivars were grown under two potash regimes in quadruplicate plots giving a total of 64 individual tuber plots and provided a range of bruise susceptibility characteristics from very susceptible to very resistant (**Figure 3.1b**). 4 plants investigated for each variety selected, taken as two plants from each of the two replicate plots. A high, medium and low susceptibility variety was selected for testing – *Cara, Pentland Crown* and *Russet Burbank*). From the previous years trials it was not anticipated that the potash nutrition treatment would significantly affect any positional variation in the plant, however to check this one set of +/- K^+ treated plants was included. Since any effect on position would be most easily observed in a susceptible variety only *Russet Burbank* with potassium nutrition treatments, were tested. All the tubers from a total of 16 plants were sampled as follows and their *in planta* distribution recorded as indicated in the **Figure 3.2** below. A reference stake (eg metre stick) was inserted close to the centre of each selected plant with the proximal soil level marked. Tubers were carefully excavated manually – each individual tuber was numbered and its approximate depth and radial position measured with a ruler (mm) and recorded. Each tuber sample was placed in a separate poly bag in a crate filled with perlite / vermiculite. After transport back to Durham the tubers were carefully place in storage at 4^{o}C until tested for impact-induced SO generation by the standard methods.

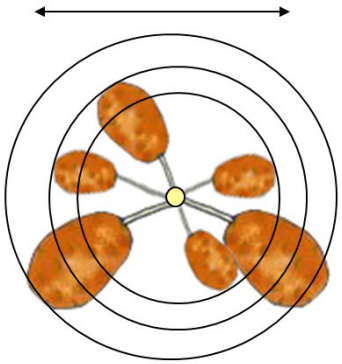
Results: The results of this study are shown as scatter plots (**Figures 3.3 a and b**).

Figure 3.2 - Positional variability within and between plants. To investigate any positional effects on bruise susceptibility accurate depth and radial measurements were taken on individual tubers from a random selection of potato plants grown at Gleadthorpe prior to assay for SO generation. Positional data were gathered from more than 100 individual tubers. The cultivars used are listed below.

a) vertical distribution – depth from soil surface



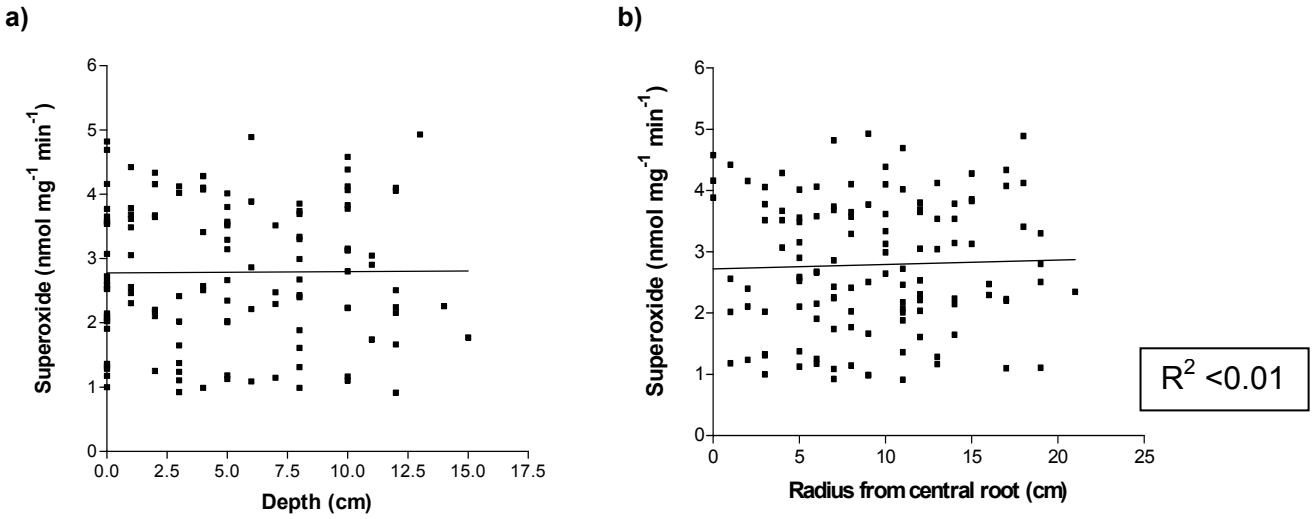
b) radial distribution – radius from centre of plant



c) cultivars sampled:

- High bruise susceptibility
  Russet Burbank (+ K^+) x 4 plants
  Russet Burbank (- K^+) x 4 plants
- Medium bruise susceptibility
  Pentland Crown x 4 plants
- High bruise resistance
  Cara x 4 plants

Figure 3.3 – Variability of susceptibility within individual plants measured by SO generation
The data show an absence of any significant correlation between position (either depth or radius) and propensity to generate SO radicals. The conclusion from this study was that it doesn't matter where on the plant the tuber is sampled from for testing. *An important qualification of this conclusion concerns earlier work in Durham which showed that any greening of the potato tubers caused by exposure of tubers to light (i.e. at or near the surface) radically decreased their bruise susceptibility. Such tubers are discarded as a matter of course.*



Conclusions: There was no correlation apparent between SO levels generated and the position of the sampled tuber in the plant (radius or depth). The Pearson R^2 coefficient was less than 0.01. Thus any tuber sampled per plant is no less representative of the population from that plant than any other. Individual tubers within a plant of any variety showed 2-7% variation in superoxide levels. Tubers within a variety show a maximum of 2-12% variation in superoxide levels (average of 8 plots). There was not sufficient time or relevance to assess the influence of tuber size outside of the normal range used by the industry for processing and packing. The effect of potassium is statistically not significant although there was a slight trend towards bruise resistance when crops were supplied with potash fertilizer. It is likely that the influence of potassium nutrition on bruise susceptibility only becomes a problem when the soil is highly deficient in this mineral which is only likely on sandy soils which would not normally be used for potato cultivation.
Recommendations: Within the limits indicated above any tuber sampled from a selected plant is likely to be representative of the crop from that plant and also from the plot. It is understood that any of the tubers sampled to be representative of a plant would be within the industry size range. Application of potash does not seem to have a significant benefit on reduction of bruise susceptibility. These findings are based essentially on investigation of potash supply in a single field (multiple plots). So this warrants further tests in

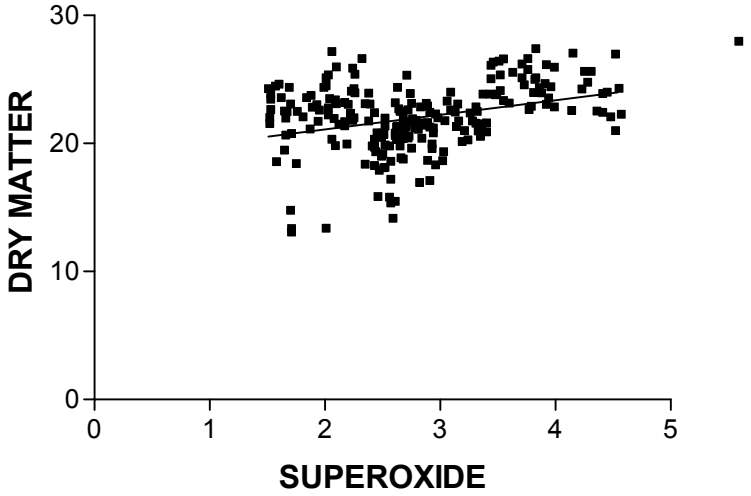
3.2 - Bruise susceptibility variation with dry matter

The estimate of dry matter is used almost universally within the potato industry as a simple measure of bruise susceptibility in which high dry matters are reported to correlate with bruise susceptibility. The underlying mechanism for this is unclear although high dry matter would be the consequence of high accumulations of starch (mainly) but also other polysaccharide materials including cell walls both of which could have an influence on the mechanical properties of tuber tissues and the damage caused on impact. Varying proportions of large to small starch grains may also influence the physical properties of the tuber tissues. The field grown material from the 2005 trials was also used to investigate the dry matter content of samples and to correlate this with bruise susceptibility/SO generation. Because individual tubers were tested a lab-scale dry matter apparatus was required. The experimental lab setup, based essentially on the industry-scale system used at McCains, is shown in **Figure 3.4a)** which enables the weight of single tubers in air and when submerged in water to be measured accurately. The dry matter was calculated from the industry standard relationship and the same tubers used for SO generation assays. **b)** shows the accumulated data for all the tuber samples. **c)** shows the data arranged into individual varieties. While there is a very weak correlation when all tuber samples were compared this is lost entirely or even inverted when tubers of the same variety were compared together.

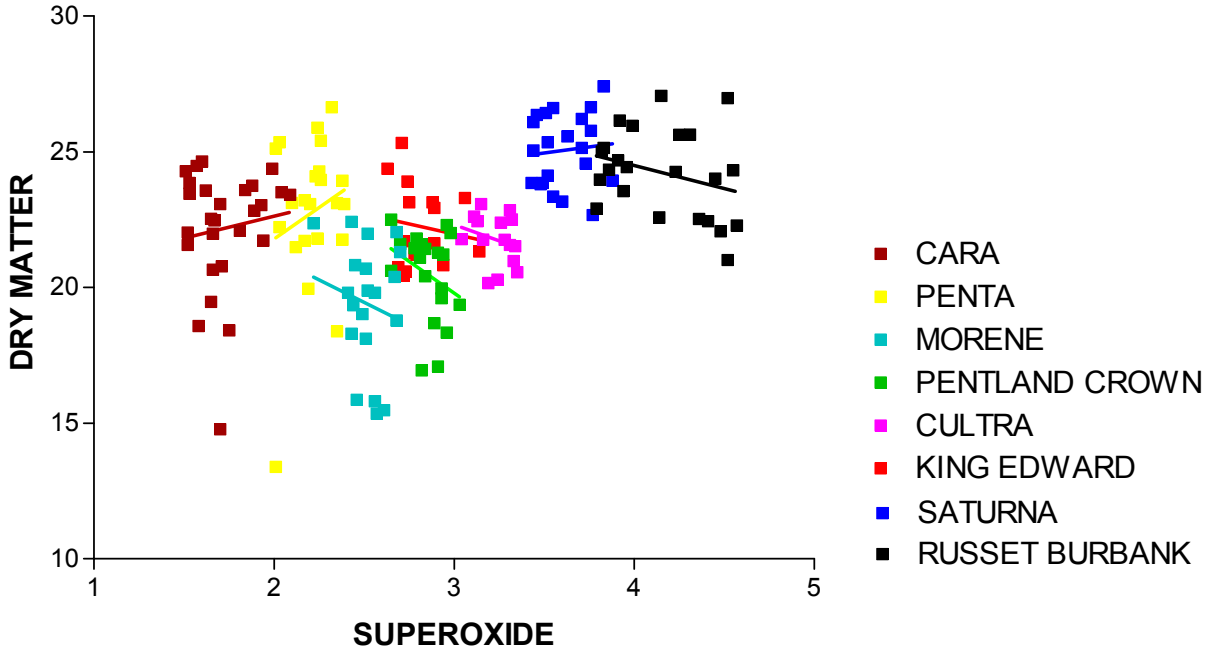
Figure 3.4 Variation of bruise susceptibility with dry matter

a)

b)



c)

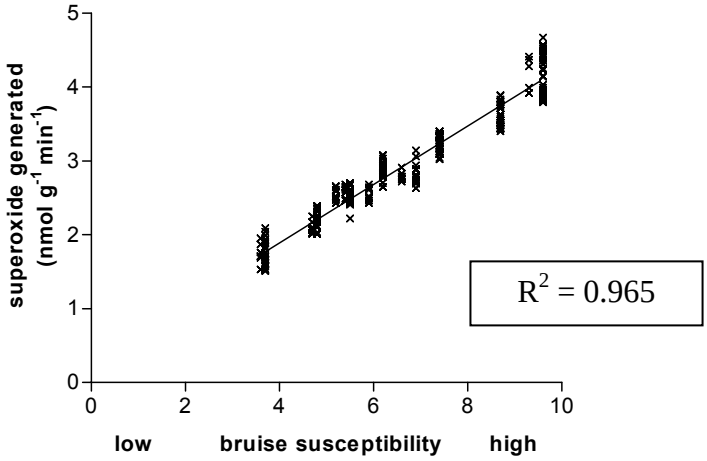


Note: Other work has indicated that cell wall pectic components (polysaccharides based around The characteristic structure of pectin is a linear chain of α-(1-4)-linked D-galacturonic acid that forms the pectin-backbone, a homogalacturonan have indicated that pectic components are responsible for rigidity in plant tissues though it is whether the amount of such pectic polysaccharides could account for the variation in dry matter would need further work).

Conclusions: There was only a low degree of correlation between dry matter and bruise susceptibility as estimated by impact induced SO generation assay (R^2 = 0.16) observed when all of the results from all varieties were plotted together. This is largely lost when tubers of the same variety are compared.
Recommendation: Consider the validity of dry matter as a parameter for estimating bruise susceptibility. The same conclusion was arrived at in other parts of the LINK project (**Section 3**).

3.3 – Cumulative correlation between bruise susceptibility and levels of superoxide generation: The 2004 trials provided a great deal of additional field grown tuber materials which was tested by the established procedures for generation of SO radicals and bruise susceptibility. The results were added to the previous data to produce **Figure 3.5** the final cumulative correlation graph.

Figure 3.5 - Cumulative correlation between bruise susceptibility and level of superoxide generation



Conclusions: The 2004 materials conformed to the previously established correlations with only a slight reduction in the Pearson R^2 coefficient but nevertheless maintaining a good linear relationship.

Part 3 - Studies on the development of field sampling methods and crop variability.

Objective 3 – Field sampling methods
Task 12 – industrial trials and materials
Task 13 – ADAS trials field variation / data handling

3.4 - Formulation of a sampling strategy for commercial assessment of bruising susceptibility:

This section was supplied by Jeremy Wiltshire (ADAS). See also **Part 3.10 - A Sampling Strategy for Commercial Assessment of Bruise Susceptibility**.

Background: Data analysis by ADAS, circulated after the consortium meeting on 6th July 2005, showed the need for more data to provide improved sample number recommendations for sampling commercial fields. These data are in the file containing the presentation given at the meeting, supplied on the accompanying CD. It was seen as important that complete data sets were obtained from as many fields as possible, to allow conclusions about a commercial sampling strategy to be based on the maximum amount of data. The following methodology, devised by ADAS was followed for the trials.

Methods, Methods and Records:

Sites: Each company should aim to provide data from three sites.

Varieties: If possible, varieties should include Maris Piper, Russett Burbank and Estima. If it is necessary to work with another variety, preferred alternatives include Lady Rosetta, Saturna, Marfona and Pentland Dell. For each field, the area sampled should be planted with a single variety.

Field size: Choose fields, or part fields, between 6 ha and 10 ha in size. Preferred field size is 8 ha.

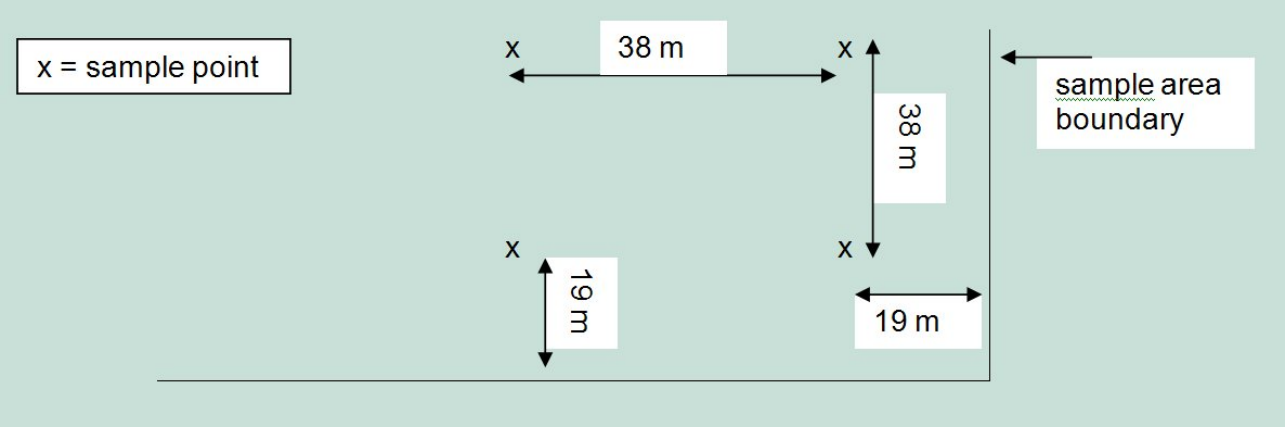
Crop management: The whole field or sampling area should be planted on (or close to) the same date. The area sampled should have been managed uniformly (i.e. the same fertiliser application rates, burn-off date, etc.).

Sampling time: Sample within two days of planned harvesting, i.e. not more than 48 h before planned harvest time.

Sampling pattern: For each field, there should be 60 sample points, with one sample of five tubers at each point. The sampling points should be spaced as evenly as possible over the sampling area. To determine the locations of the points, do the following:

1. Calculate the area of the sampling area in square meters (m^2). This is the area in hectares multiplied by 10,000.
   E.g. Sampling area = 8.5 ha, $8.5 \times 10{,}000 = 85{,}000\ m^2$
2. Divide the area (in m^2) by 60, to give the approximate area per sample.
   E.g. for an area of 85,000 m^2, $85{,}000 / 60 = 1417\ m^2$
3. Take the square root of the area per sample to give the approximate distance between sample points in each direction, and round this to the nearest whole metre.
   E.g. for an area per sample of 1417 m^2, $\sqrt{1417} = 37.6$ m (rounded = 38 m)
4. Make a sketch map of the sample area and mark the approximate positions of the sample points. Distance from the edge of the field/sample area to the closest point should be half the distance between adjacent points.
   E.g. if the distance between points = 38 m (as in above example), distance from boundary to nearest point = 38/2 = 19 m. See diagram in **Figure 3.6** below, for this example.
5. Locate sample points in the field by pacing between points or by counting rows (after calculating the number of rows in the distance between points). Approximate as required to ensure that there are 60 sample points. It may help to mark all the points with canes in advance of the sampling operation.

Figure 3.6 – Field sampling strategy 2005-6 season



Sampling method: Make a sketch-plan of the field, showing the arrangement of sampling points. Record soil temperature at tuber depth, (one measurement for the field). Sample 5 tubers per sampling point, each from a different plant. Tubers should be within the size range 60-70 mm. Take the tubers to a laboratory and assess bruise susceptibility using the test kit provided, following instructions provided by the University of Durham. Assess the tubers in groups of five, giving one measurement per sampling point. This should be done by taking one core from each of the five tubers, and placing all five cores in the same container, as described in the instructions provided by the University of Durham (See **Appendix 1 – Instruction Manual 2005**). Record tuber dry matter, if possible one measurement for each sample of five tubers. Use the Excel spreadsheet to store the data.

Data recording: Three Excel spreadsheets are provided within the document file to allow data to be stored in a common format. Use a different copy of the spreadsheet for each field, each saved with a different filename. Important variables include:

1. field location
2. soil type
3. variety
4. intended market
5. planting date
6. burn-off date
7. harvest date
8. area sampled (ha)
9. fertiliser applications
10. irrigation events (dates and mm of water applied)
11. commercial assessment of bruise susceptibility using the barrel method at store intake (or grading if the crop will not be stored)
12. dry matter of each sample
13. colorimeter reading for each sample

It is essential that the results of any conventional bruise tests performed on these crops is also recorded in these datasheets also.

Data processing: All data to be sent to jeremy.wiltshire@adas.co.uk for analysis.

3.5 Planning field work and data analysis, 2005:

Relationships: This work will be done by four industrial partners (Branston, Greenvale, McCain, MBM) with assistance from ADAS. ADAS will provide the experimental protocols (See **Part 3.4 - Commercial field trials 2005. Formulation of a sampling strategy for commercial assessment of bruising susceptibility**) to ensure that all work is done in a consistent way in order to allow rigorous statistical analysis. ADAS will analyse the data, provide a written summary of the results and present the results to the consortium.

Relationships between bruise index and dry matter: This relationship can be tested using the data collected in the work described below.

Spatial variation: Each company will provide two sites. Varieties should include Maris Piper, Russett Burbank and Estima. Each field will be sampled at 20 points, 3 samples per point, giving a total of 60 samples per field. This test will be performed just before harvest. Bruise susceptibility assessment will be done after transport of the tubers to a laboratory. Records should include dry matter of each sample, all crop husbandry, and a commercial assessment of bruise susceptibility using the barrel method at store intake.

Temporal variation: Earlier work has shown considerable temporal variation. Further work is needed to check variability between sample times approaching harvest. This work could be carried out within the same fields used for the spatial variation work (see above). Bruise susceptibility will be assessed on two occasions, the first at least one week after defoliation, and the second (also used for spatial variability analysis) as close as practical to harvest. The interval between sample occasions will be 7-10 days.

ADAS Costs: This work is extra to the work planned within the LINK project, so additional funding will be required for the time inputs by ADAS staff. These extra costs were met by BPC.

3.6 Variation in potato bruising and implications for sampling:

The original report on this part of the project was submitted to the consortium in 2006.

Introduction: An understanding of the degree and nature of spatial and temporal variation in potato bruising within fields is useful for development of the practical use of the Durham test kit. Current understanding of the causes of bruising in potatoes suggests that significant variation may exist within fields. This variation may be caused by processes that act spatially within fields, such as variation in soil nutrients and soil water availability. Other processes that occur randomly within fields, such as seed condition, may also be important. Data collected in 2004 and 2005 were used to provide sampling recommendations for use of the bruise susceptibility test kit. These data were used alongside the views of the consortium on the practical (time and materials) limits to commercial sampling, and the confidence level required. Such data also have value for bruising assessments using traditional methods (barrel, hot box and peeling).

Objective: The objective of this part of the project was to evaluate within-field spatial variation in potato bruising and implications for sampling with the Durham test kit.

Approach: The work involved five components, and the results are presented under these headings:

- spatial variability in bruise index within potato fields;
- links between spatial variation in bruising and soil properties;
- links between variation in bruising and tuber dry matter;
- temporal changes in bruise index;
- a sampling strategy for commercial assessment of bruise susceptibility.

3.7 Spatial Variability In Bruise Index Within Potato Fields:

Materials and Methods: Five commercial fields were studied in 2004. The area in each field was an 8 ha block (2 ha x 4 ha), with a sample location on each intersection, giving 15 sample locations. At each location, 5 plants were sampled. Three plants were sampled from the same row (each separated by one un-sampled plant), and one plant was sampled from each of the adjacent rows. Distance between plants was not more than one metre. From each plant 5 tubers were sampled. Tubers were selected to be in the middle of the tuber size distribution. Assessment of bruise index followed the instructions provided with the kit. A single core was taken from each tuber, and cores from five tubers from a single plant were put into a single vial, so one measurement per plant was obtained.

Results: A summary of the results is given in **Table 3.1**. The main results from each of the consortium partners are plotted as histogram graphs in **Figures 3.7** to **3.11**.

Table 3.1 - Summary of bruise index results for each of the 5 field sites				
Site	**Cultivar**	**Minimum**	**Mean**	**Maximum**
Branston	Saxon	2.45	2.95	3.50
R. Holme	Maris Piper	2.00	3.18	6.00
Spalding	Maris Piper	2.95	4.43	6.60
Lincoln	Russet Burbank	3.70	4.65	5.45
Fakenham	Saturna	4.25	5.71	7.45

The five sites demonstrated differing patterns of variability. The Branston site showed relatively low bruise index values and variability (**Figure 3.7**). This was also the case at Runcton Holme, with the exception of one high value (**Figure 3.8**). In contrast, sites at Lincoln and Fakenham showed greater bruise index values and greater variability (**Figures 3.9 and 3.10**). At Spalding there was some systematic variability related to location (**Figure 3.11**). To a lesser extent, there was some indication of systematic variability at Fakenham, but at Branston, Runcton Holme and Lincoln the variability was random with respect to location.

Figure 3.7 - Bruise index values, Branston site

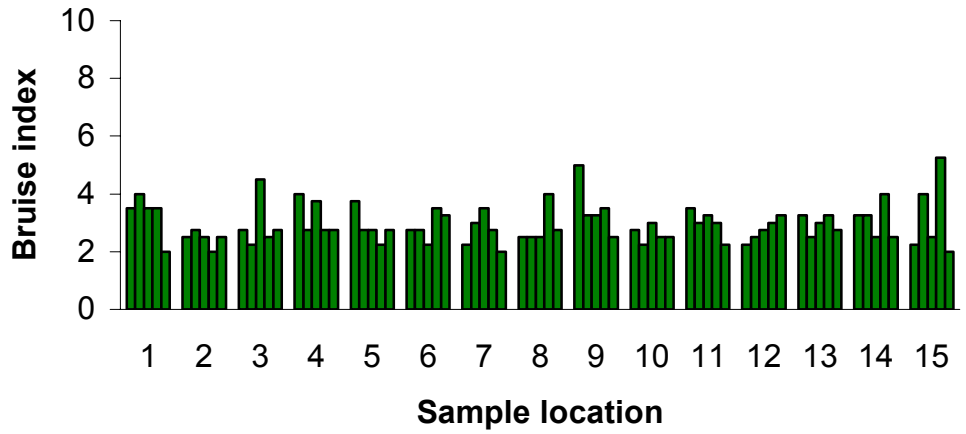


Figure 3.8 - Bruise index values, Runcton Holme site

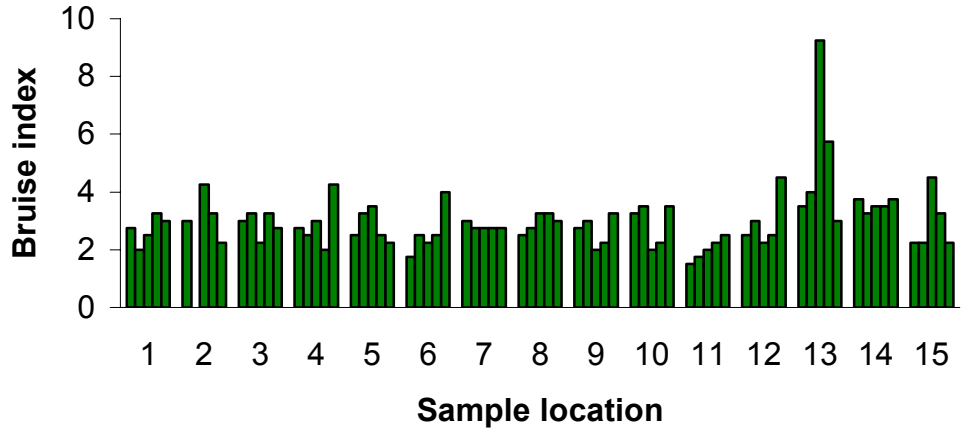


Figure 3.9 - Bruise index values, Lincoln site

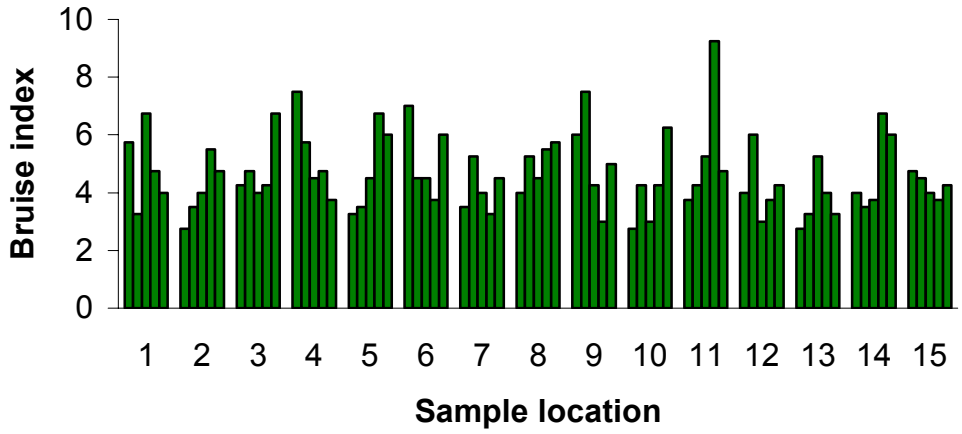


Figure 3.10 - Bruise index values, Fakenham site

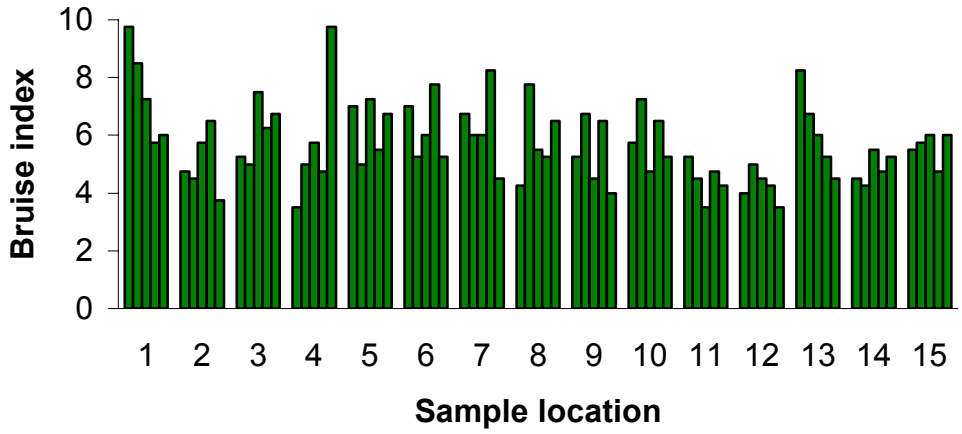
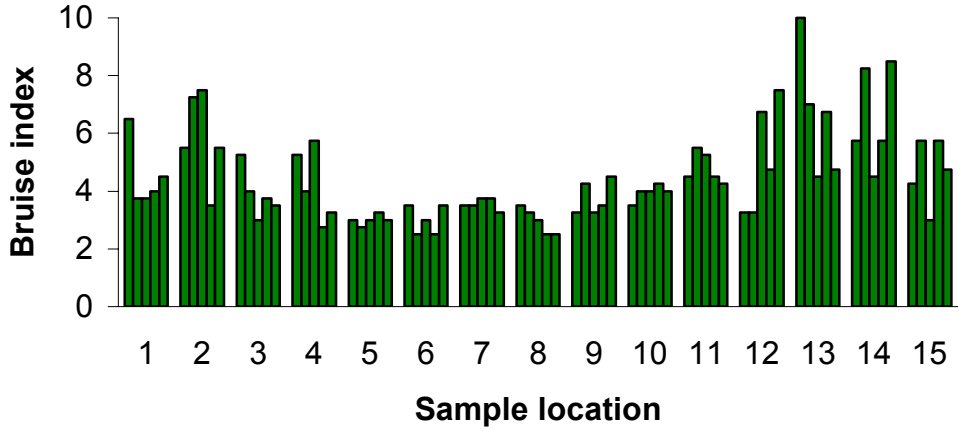
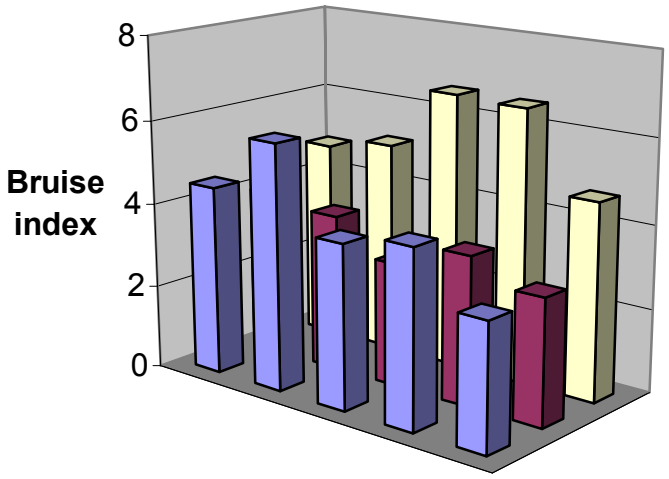


Figure 3.11 - Bruise index values, Spalding site



The data for the Spalding site are presented in **Figure 3.12**, in two dimensions, showing the way the locations were arranged in the field. This shows that the higher bruise indices were grouped together along one side of the field. Examination of the field indicated that irrigation practice was a possible cause of this.

Figure 3.12 - Mean bruise index values for each sample location, Spalding site. Bars are arranged in the same spatial pattern as the sample locations



Discussion: The data presented above provide information on the characteristics of spatial variability in bruise index. Bruise index is a measure of superoxide production, which is related to bruise susceptibility (Johnson *et al* 2003). Thus, these data have relevance for bruise susceptibility. That widely-differing patterns in bruise susceptibility were found in this study of only five crops, suggests that the extent and pattern of variability is highly variable between crops. Before sampling a potato crop to assess bruise susceptibility, a grower will not know what the pattern of variability is. Thus, a sampling strategy will need to be applicable to a wide variety of variability scenarios. Further work to develop a sampling strategy is presented later (See **Section 3.10** and also **Section 3.4**).

3.8 Links Between Spatial Variation in Bruising and Soil Properties:

Methods: Five commercial fields were sampled in 2004 for assessment of bruise susceptibility as described in **Part 3.7** above. At each of the 15 sample locations a soil sample was taken, from just below tuber depth, to determine soil P, K and Mg content and soil pH. The data were used to compare these soil chemical properties with bruise susceptibility.

Results: For the data set as a whole (75 sample points amalgamated from the five fields), there were relationships between soil pH and bruise index, and soil phosphorus content and bruise index (Table 2). However, these correlations were not found to be significant for individual fields.

Table 3.2 - Correlation coefficients between bruise index and soil factors

	Soil factor			
	P	pH	K	Mg
Pearson Correlation	-0.276	-0.473	-0.062	0.103
Significance (P)	0.017	<0.001	0.596	0.379

Discussion: It was concluded that there was not a relationship between soil analysis results (P, K, Mg and pH) and bruising index that could be used to help indicate bruise susceptibility risk.

3.9 Links Between Variation in Bruising and Tuber Dry Matter:

Methods: Data were collected as described in **Part 3.11** below (see Methods, 2005).

Results: The relationship between bruise susceptibility and dry matter was very poor (Table 3). For the site with the best relationship, the variance in bruise susceptibility accounted for by dry matter was 5%.

Table 3.3 - Relationships between bruise index and dry matter for nine crops in 2005

Crop	Variety	*Regression equation*	% variance accounted for
1	Estima	Index=0.453×DM-4.46	5.0
2	R.Burbank	Index =-0.426×DM+13.65	3.6
3	M.Piper	Index=0.0391×DM+3.54	0.0
4	M.Piper	Index=0.0502×DM+3.43	0.0
5	M.Piper	Index=0.128×DM+1.12	0.0
6	Sante	Index=0.277×DM+0.15	4.6
7	R.Burbank	Index=-0.187×DM+10.33	0.0
8	M.Piper	Index=0.065×DM+3.52	0.0
9	R.Burbank	Index=0.164×DM+2.12	0.0

Discussion: Anecdotal information from industry sources has suggested that the risk of bruising is related to tuber dry matter content, with high dry matter values indicating high risk. The data presented here do not support this view.

3.10 Temporal Changes in Bruise Index:

Methods: For six fields in 2004, five samples points were marked, evenly spaced across the field in a diagonal line. two samples of five tubers were taken at each point and analysed using the test kit for bruising index. this was carried out at four times:

1. two weeks before haulm destruction;
2. at haulm destruction;
3. two weeks after haulm destruction;
4. at harvest.

Results: The pattern of change between times of bruise index assessment varied between fields. For example, bruise index values for two fields are shown in **Figures 3.13** and **3.14**. The changes between sample occasions were large (up to 4 bruise index units).

Figure 3.13 - Temporal change in bruise index in a crop of cv. Cara in 2004

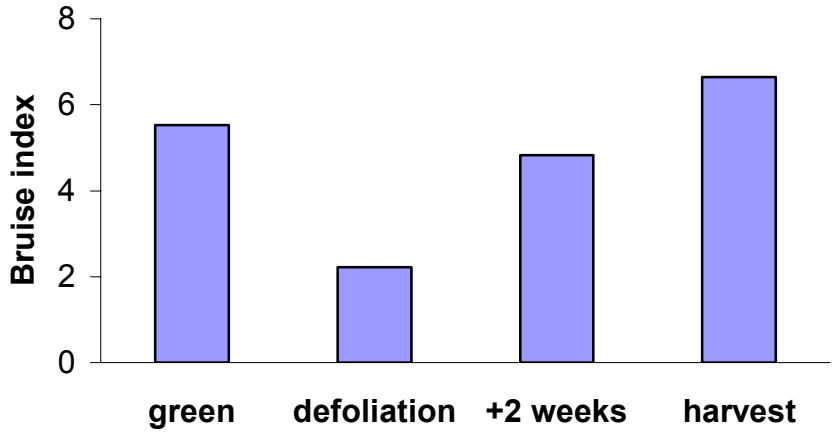
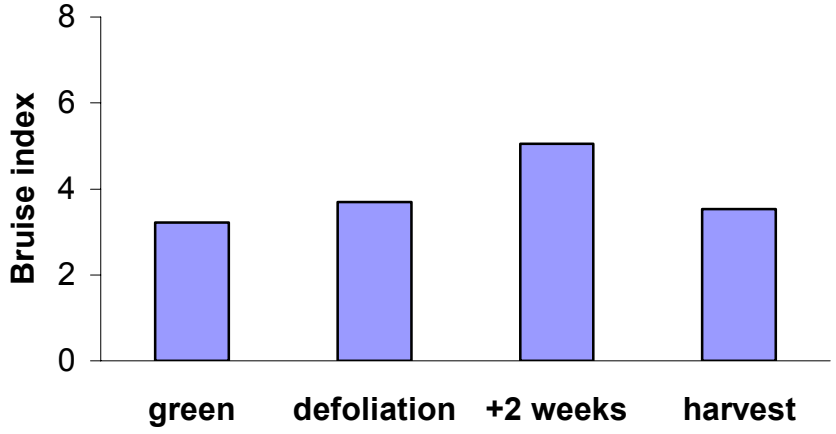


Figure 3.14 - Temporal change in bruise index in a crop of cv. Russet Burbank in 2004



Discussion: The results show that it is not possible to estimate bruise susceptibility at harvest by sampling two weeks or longer before harvest.

3.11 A Sampling Strategy for Commercial Assessment of Bruise Susceptibility:

Methods, 2004: Data were collected as described above in **Part 3.6**. **Spatial Variability In Bruise Index Within Potato Fields**. Data were collected from five sites, each a block of 8 ha (200 m × 400 m), with sampling locations at each intersection point on a 100 m grid, including the grid edges, giving 15 sampling locations. At each location, five plants were sampled, and five tubers from each plant. For each plant, a single measure of bruise susceptibility was obtained using the testing kit. Thus, 75 values per field were recorded. For each field, data were subjected to analysis of variance, using the 15 locations as plots and the five samples at each location as replicates.

Methods, 2005:

Sites: Each company provided data from three sites. The sites are listed in **Table 3.5** by data provider (company) and variety.

Crop Management: For the sampling areas selected, the whole field or sampling area was planted on (or close to) the same date. The area sampled was managed uniformly (i.e. the same fertiliser application rates, burn-off date, etc.).

Sampling Time: Samples were taken within two days of planned harvesting.

Sampling Pattern: For each field, there were 60 sample points, with one sample of five tubers at each point. The sampling points were spaced as evenly as possible over the sampling area.

Sampling Method: At each sampling point 5 tubers were taken, each from a different plant. Tubers were within the size range 60-70 mm. They were taken to a laboratory and bruise susceptibility was assessed using the test kit provided by the University of Durham, following instructions included with the kit. The Tubers were assessed in groups of five, giving one measurement per sampling point. This was done by taking one core from each of the five tubers, and placing all five cores in the same container, as described in the instructions provided by the University of Durham (**Appendix 1 - Blackspot Detect Instruction Manual 2005_2006**). On nine of the 12 sites, tuber dry matter was recorded for each sample of five tubers.

Results: In 2004, for some sites (Spalding, Runcton Holme and Fakenham) the variability within locations was less than the variability between locations. This was especially marked for the Spalding site. At the other two sites variability was similar within and between locations. At each site the standard deviation was calculated using all 75 samples. This was then used to calculate the number of samples required to create confidence limits such that these limits were no more than 1 bruise index unit away from the sample mean. This number was calculated for 3 confidence levels: 95%, 97.5% and 99% (**Table 3.4**).

Table 3.4 - Data collected in 2004: mean, range and standard deviation (SD) of bruise index at each site. Number of samples needed to give a range<(mean+/-1) at 95% confidence level (N1), 97.5% confidence level (N2) and 99% confidence level (N3).

Site	Mean	Range	SD	N1	N2	N3
Spalding	4.43	2.50–10.00	1.57	12	16	21
Runcton Holme	2.99	1.50–9.25	1.04	7	9	11
Branston	2.95	2.00–5.25	0.67	5	6	7
Fakenham	5.71	3.50–9.75	1.36	10	13	17
Lincoln	4.65	2.75–9.25	1.29	9	12	15

The data in **Table 3.4** show, for instance, that for sites such as Fakenham with a mean of 5.71 and an SD of 1.36, if 10 samples were taken, the 95% confidence limits would be 5.71 +/- just under1. Thus, we would expect 10 samples to be sufficient to produce the required accuracy in 19 sites out of 20. Similarly, 13 samples would be needed for the 97.5% confidence level (sufficient accuracy in 39 sites out of 40) and 17 samples needed at the 99% confidence level (sufficient accuracy in 99 sites out of 100).

For sites where variability within locations was significantly less than the variability between locations, these figures are an under-estimate of the number of samples required, because the standard deviation is smaller than would have been the case if samples had been independent (not grouped). Data collected in 2005 were not grouped for this reason.

A summary of the analysis of data collected in 2005 is given in **Table 3.5**. This shows the mean, range and standard deviation (SD) of bruise index at each site, and number of samples needed to give a field mean at five different levels of precision (+ or - 0.5–1.5 at increments of 0.25), and at a 95% confidence level.

The target level of precision selected by the LINK consortium was +/- 1 bruise index unit. Inspection of **Table 3.5** shows that the most variable field in 2005 (the ninth field in **Table 3.5**) required 9 samples for a

value within 1 unit of the field mean on 19 of 20 occasions. Six of the 12 fields required 5 samples or less for a value within 1 unit of the field mean on 19 of 20 occasions.

Table 3.5 shows how the number of samples required increases greatly if greater precision is required. For example, for the most variable field requiring 9 samples for a value within 1 unit of the field mean, if a value within 0.5 units of the field mean is required, then 34 samples are needed.

Table 3.5 - Data collected in 2005: mean, range and standard deviation (SD) of bruise index at each site. Number of samples needed to give a field mean at five different levels of precision (0.5–1.5 at increments of 0.25), and at a 95% confidence level.

		Bruise index			Number of samples needed to give field mean +/-				
Data provider	**Variety**	**Mean**	**Range**	**SD**	**0.5**	**0.75**	**1**	**1.25**	**1.5**
Greenvale	*Estima*	3.92	2.48-7.13	1.030	17	8	5	3	2
	R. Burbank	3.52	2.17-6.51	0.826	10	5	3	2	2
	M. Piper	4.37	2.79-6.05	0.859	12	6	3	2	2
Branston	*M. Piper*	4.67	2.95-6.20	0.783	10	5	3	2	2
	M. Piper	3.54	1.71-7.13	0.938	14	7	4	3	2
	Saxon	4.63	1.86-7.91	1.208	23	10	6	4	3
McCain	*R. Burbank*	6.35	3.87-10.54	1.382	30	14	8	5	4
	M. Piper	4.98	2.17-10.23	1.357	29	13	8	5	4
	R. Burbank	5.32	3.26-9.15	1.469	34	15	9	6	4
MBM	*P. Dell*	4.11	2.17-9.61	1.155	21	10	6	4	3
	P. Dell	4.53	2.02-8.06	1.341	28	13	7	5	4
	Hermes	5.51	3.88-7.29	0.801	10	5	3	2	2

Conclusions:

1. The ADAS field trials have suggested that to predict bruise susceptibility to 95% confidence and to within +/- 1 bruise index unit then 5 to 9 samples per field are required.

2. From the one experiment carried out by Greenvale and ADAS there appears to be no direct correlation between traditional bruise testing methods and the new bruise testing kit. There is high variability in both tests though no correlation between the two methods.

Recommendations:

From the statistical analysis of the 2005 data ADAS propose 4 recommendations:

- Identify the sampling area with care. Areas with different crop management, or different soil type should be treated as separate sampling areas.
- If variety and season suggest a low risk, take 5 samples per field.
- If variety or season suggest a high risk, take 10 samples per field.
- If average bruise index of 5 samples is >5.5, or any single value is >7.5, this would suggest either high bruising risk or high variability, so re-test the field with 10 samples.

3.12 Field trials 2006 - Extension of the LINK project

The following reports do not form a part of the original LINK project but represent extra work continuing the field evaluation of the Blackspot Detect kit. The work was carried out during 2006 after the official period of the LINK project and was financed by the individual companies participating in the trials and by BPC. The results were reported by Robert Blades of McCains Foods (GB) Ltd, Andy Barker of Branston Potatoes Ltd and Adrian Neil of Greenvale AP Ltd. The short reports are included here as an important part of the commercial and field evaluation of the kit by the prospective end-users. Further evaluation work carried out by Cambridge University Farms (CUF) and Scottish Agricultural College (SAC) were funded by BPC and which resulted in substantial and relevant reports but which were deemed inappropriate to report here. Only the main conclusions from these projects are reported here as they relate to the projected commercialization of the kit.

McCains - Bruise Detect Susceptibility Kit 2006

The LINK project finished last year however the commercial partners agreed to another season in using the now fully functional kit on 10 commercial fields each.

Sample collection: samples were collected from fields within planned harvest of 48 hours. Method of sampling was 5 sample points per field. 1 tuber per 5 different plants equalled a sample. A duplicate sample was taken per point to be used to determine the 'Damage Index'

Field samples were collected one day and then kept at ambient temperatures till the following day to carry out the impacting. Fields 1 to 6 were collected on the 24 October, Fields 7 to 10 collected on 1 November, 2006.

The Bruise index was determined following the kits instruction manual and the Hana bruise index meter instruction manual.

The duplicate sample was then used to determine the Damage Index by the following:

- Impacted the tubers at the stolon end using the impactor from the kit
- Placed tubers in hotbox at 20^{o}C for 24 hours depending.
- After hotboxing tubers inspected and divided into categories – Undamaged, Scuffed, Slight, Severe,

" Scuffed" – is defined as broken skin only with no flesh damage.
" Slight" – is defined as flesh damage removable by 2 strokes of a peeler.
" Severe" – is defined as damage not removed by 2 strokes of a peeler.

Once assessed, the weight of tubers (a) in each category was recorded. From this the Damage index was calculated using the following table.

Table 3.6 – Sample collection 2006, categories and damage index calculations

Damage Type	Weight of Tubers	% Total Weight	Factor	Damage Index
Undamaged	(a)	(c) = (a/b * 100)	0	= (c) x factor
Scuffed			1	
Slight			3	
Severe			7	
Total	(b)			(d)

Finally, of the fields that have been delivered into the factories so far the levels of Old Bruise were assessed, All Damage (old, new and mechanical damage) have also been included for information. For a tuber in a sample to be categorised as defective it needs to be the following' if the defective portion is removed with a

straight cut, the defective portion will represent 5% or more of the total weight of the tuber.' 10% of a defect in a sample would be considered 'rejectable'.

Results:

Table 3.7- Summary of data and damage indices for 10 fields

Field	Variety	Bruise Index	Range		Damage Index	Intake Old Bruise	All Damage
1	R Burbank	4.72	3.41 - 7.11	Moderate	162.2		
2	R Burbank	3.57	3.04 - 3.97	Resistant	200.7		
3	R Burbank	3.50	3.11 - 3.81	Resistant	143.9		
4	R Burbank	4.08	3.05 - 6.63	Resistant	103.6	0.25	0.85
5	R Burbank	3.98	3.64 - 4.76	Resistant	100	0.40	1.65
6	R Burbank	2.98	2.67 - 3.48	Resistant	100		
7	R Burbank	5.46	3.78 - 6.60	Mod/Susceptible	269.4		
8	R Burbank	6.91	6.09 - 8.15	Susceptible	297.6		
9	P Dell	6.31	4.69 - 7.94	Susceptible	208.4	0.71	1.28
10	P Dell	6.56	4.82 - 9.49	Susceptible	268.9	1.20	10.2

Discussion: On the positive side this was the best year in testing for the kit as a tool, the Hana meter gave a lot more confidence than the previous colorimeter. On the down side the environment for doing the test has to be fairly sterile particularly when preparing the cuvette for the readings, fingerprints etc.

Certainly the year would not be considered a high risk for bruising this is backed up for the low levels of the delivered crops so far in the results table and looking at other material that has been delivered into factory and store.

The results between the bruise index and the damage index are disappointing; there was no correlation between the bruise index and the damage index. The reason for this needs to be determined before any future plans for the kit can go forward.

Branston comments and results summary:

As Robert has pointed out, this was not a problem year for bruising. We had no specific crops that were a particular problem, more a case of poor harvesting management.
While running the tests, there were no obvious correlations between the different techniques/protocols and this is perhaps borne out by the results.

We appreciate the idea and the potential behind the approach but have some comments:

The damage index could be seen as a bit subjective, particularly between operatives and when in a hurry to complete a set of measurements. It is a time of year when time is at a premium. (I eventually left out the 'scuffed' category for simplicity.)

The time to take and run the test is too long:

> If a field is approximately uniform in soil type, can we just not take one sample but with more tubers perhaps?
> Could we take one tuber from ten random spots across a field and treat as one sample?
> Can we shorten any of the steps?

Table 3.8 – Summary of data and damage indices for 9 fields - Branston

Soil type	Variety	Irrigated	Bruise Index	Range	Damage Index	Comment
Silt loam	Marfona	No	4.9	4.2-5.6	184	
Silt loam	M.piper	No	4.08	3.2-4.5	220	
Silt loam	M.piper	No	5.4	4.6-6.2	172	
Silt loam	M.piper	No	4.6	3.5-5.4	114	
Sandy loam	Estima	Y	3.9	3.2-4.5	100	
Sandy loam	Saxon	Y	3.5	2.5-4.0	100	
Med sandy clay loam	Saxon	Y	3.7	3.1-4.6	110	
Med sandy clay loam	M.piper	Y	3.5	2.5-4.0	124	
Sandy clay loam	Desiree	Y	4.8	2.3-5.7	150	

Greenvale - Black spot detect Bruise susceptibility Assessment 2006.

This year Greenvale carried out a test of the kit using its agronomists at two sites one in Norfolk and one in Shropshire.
Rob Blades and I conferred and agreed on a protocol for the commercial test, which Rob then produced and circulated.

The kit was then given to the agronomists along with the protocol to follow after some initial instruction from myself. I will not repeat the protocol as it is included in Robs report and a copy is attached.
Agronomists were asked to sample fields that they could monitor and sample 48 hrs before harvest. Greenvale tried to be ambitious and had two kits so that we could cover two areas. Unfortunately the late arrival of the colorimeter limited us starting at both sites until I borrowed the colorimeter from CUF. No tests were carried out with the new colorimeter.
Two samples were taken at each of the 5 points in the field one to be tested using the kit and one to be impacted and hot boxed.
Agronomists were asked to carry out a follow up assessment of the crops once harvested either by factory intake QC, Hot boxing samples off harvester or ex store.

Results
The results are shown on the attached spreadsheet.
Initially samples tested by the kit did seem to mirror results from the hot box but this appears to be where the test showed susceptible the hot box developed a very high index indeed. Others showed very little correlation between the two tests. In all cases the prediction of bruising susceptibility was not reflected in the harvested samples. It became apparent that the impactor was possibly bruising the potatoes too much and that the impact did not reflect actual harvesting conditions.
This was the first year we let the agronomists have ago themselves and as a result only 10 out of 20 fields were done as they found the kit time consuming to put into practice. They have also not completely followed through consistently with the follow up information. What is apparent though, is where bruising indexes were recorded by factory QCs they are significantly below the indicated indices when using the impactor.
One sample had to be aborted due to contamination of soil/skin material after a poor washing. This is a vital area of control that can be difficult to do without the right room and facilities.

General comments: -

- Agronomists found the impactor and corer easy to use as a stand-alone piece of equipment.
- The time element was significant in reality they would find it easier to sample 50tubers impact in the field and hotbox overnight. As this needed less cleaning and lab time.
- Agronomists found maintaining timing and hygiene difficult during high pressure work periods
- Because we were still using the old colorimeter there was still variation in readings if the tube moved slightly.
- It is still possible that the impactor is exaggerating the susceptibility

Table 3.9 – Summary data for 9 fields comparing kit prediction and hot box assessment - Greenvale

Site/grower	Variety (average kit index)	Blackspot Kit bruise prediction	Hot box result average	Bruising index	Post harvest bruising report	Correct prediction?
Hall Farm Suffield	**Maris Piper**					
	5.8	SUS	18.2	264	4% Moderate 8% slight	☑
Rookery Farms	**Estima**					
	4.4	RES	17.8	28	No bruising recorded on analysis. 1% mechanical damage	☑
Stearn Alston	**Estima**					
	4.74	MOD	17	76	Store analysis recorded no bruising issues	☒
Bubb Musta Hill	**M Piper**					
	6.44	SUS	21.1	224	some minor bruising index 126	☑
Brown Kempters	**M Piper**					
	7.64	SUS	-	236	Post harvest bruising index 26	☑
EJ Andrews	**M Piper**					
	4.6	MOD	22.8	244	Hot boxing at harvest showed an index of 140	☒
W Wrinch	**M Piper**					
	4.7	MOD	22.5	440	No issues recorded re bruising	☒
Bubb Reservoir	**M Piper**					
	3.98	RES	19.6	0	No problems Bruising index 83 and 68 recorded	☑
Davies Morton Corner	**M Piper**					
	9.2	SUS	-	304	No data	☑

Comment: The last column in **Table 3.9** was added based on the colour scheme in columns 3 and 5.

Part 4 - Commercialisation

Objective 4 – Commercial exploitation

Commercial Development

On commencement of the project, a commercialisation subgroup was set up to provide the commercial steer to the project. Chaired by the commercialisation partner, Gavin Lishman of Martin Lishman Limited, this group also included a member from BPC (Sue Cowgill, Kate Jackson and Ewan Brierley) and from the Durham University Technology Transfer Office (Tim Hammond).

4.1 Markets:

At the outset of the project, BPC provided information on producers and scale of cultivation This is illustrated in Table 1. Whilst the numbers of producers are known to have reduced during the course of the project, the general messages are believed to be still relevant.

Table 4.1 2001 BPC Producer data

Cultivated Area – from	Cultivated Area - to	producers	total area	percent total area
0.1	1.99	611	737	0.526289
2	3.99	665	1945	1.388919
4	5.99	481	2388	1.705264
6	7.99	355	2591	1.850225
8	9.99	356	3284	2.345095
10	11.99	273	3110	2.220842
12	13.99	230	3122	2.229411
14	15.99	156	2444	1.745253
16	17.99	168	2993	2.137292
18	19.99	156	3093	2.208702
20	24.99	304	7117	5.082228
25	49.99	780	29119	20.79379
50	74.99	307	19589	13.98845
75	99.99	154	13761	9.826689
100	199.99	170	24377	17.40754
200	399.99	57	20367	14.54401
	TOTAL	**5223**	**140037**	

Not unexpectedly, the shaded area of the Table demonstrates that 76% of the area is cultivated by 28% of the producers (1468). The target market for the potato bruising kit was determined to be the in-house and contracted agronomists working for these larger producers and the agronomists working for the processers and packers as represented by members of the LINK consortium.

During 2004 BPC commissioned a bruising survey which was distributed to 3500 growers, processors and packers. This survey posed questions on bruising in general and then followed up with specific questions related to the provision of a risk assessment tool and the requirements and price acceptability to the market.

The key points that were fed back which were of relevance to the kit were:

- average bruising losses were an average of £200/ha (and as high as £1200/ha)
- on a 50ha holding this equates to £10,000
- total losses to industry from bruising were estimated at £30m/yr
- 85% of respondents said they would like a practical risk tool
- 82% of respondents were growers
- c.80% of respondents said they would consider a 2 hour bruising test
- On pricing, only one respondent mentioned a price of £400, for several others price was not an issue.

These findings strongly imply that the kit would be a well received tool for use by the grower on the farm. It was agreed that presenting the kit price as a percentage of the annual loss due to bruising would be an effective way to justify a final selling price

4.2 Cost model for *Blackspot Detect* test kit and consumables: Throughout the research programme, cost models have been developed to reflect the preferred configuration of the test methodology. These have been updated in light of the technical developments outlined in this report.
The test protocol has been outlined in **Part 1.4** and the latest protocol is described in **Appendix 2 – Instruction Manual**. The costing has been broken down into the three key components: impactor, chemistry and spectrophotometer. A key objective has been to take the designs and materials from the research programme and to scale up for bulk manufacture in the most cost-effective format. This has necessitated sourcing raw materials, consumables and specifying instrument design.

4.3 Hardware – impacter and corer: As highlighted earlier in the research report, successive modifications have been made to simplify the bruise and core cutter designs to minimise the number of moving parts and to simplify the design for manufacture. From an design point of view, the initial design which was essentially a "gun" format was felt to have potential issues with respect to enhanced security measures, for example if carried in luggage for air travel. A simplified barrel design which retained full functionality was therefore produced. Martin Lishman Limited have taken the Durham designs and worked closely with a local engineering company to reduce the number of working parts and to simplify the machining and assembly process for the finished impactor and designs . In doing so, costs have been reduced from over £300 to around £55 for a small production run of the impactor and core cutter. These changed designs were submitted for evaluation by the Durham research team who confirmed that the functionality of the devices was maintained.

4.4 Chemistry: The key chemical process may be summarised by the simple operations shown opposite -

- Pour tube (2) into bottle (1) to make solution.
- Add cores
- Wait 20 mins
- Snip off nozzle top
- Pour into reading tube
- Read in colorimeter

Bottle 1) comprises an Aslon bottle containing the assay chemical, XTT. As the chemical was not stable in water, the most practical route for application into the Aslon bottle was to disperse from an emulsion in ethanol and to use a micropipette to deposit 10 microlitres in the bottle. This was then dried down to leave a powder deposit in the base of the bottle.

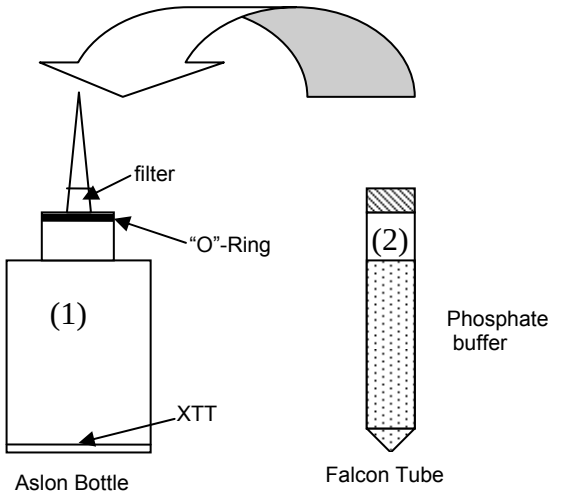


Figure 4.1 Key chemical process

XTT is a complex tetrazolium compound as defined in **Appendix 6** and in **Part 1**. The product was initially sourced through Sigma Aldrich at a catalogue price of around £624 per gramme. As 0.8mg is used per test, this approximates to a material cost of 50p per test. To reduce costs, the original manufacturer of this material was traced to a Canadian company, Biovectra DCL. Biovectra custom manufactures the material in house as a reagent for the assay of cell structures. Their standard production batch size is 820g and the product is considered to be a routine product with good security of supply as the material is sold on to many customers.

The pricing depends on the volume purchased. **Table 4.2** calculates the cost per test based on US dollar prices at the 3 key price breaks using an exchange rate of 1USD to £0.491 and the application of 0.8 mg of the reagent per bottle.

Table 4.2 XTT raw material costs		
Minimum quantity / g	**Price per gram**	**Cost per test**
1	$325	£0.128
100	$182	£0.071
1000	$133	£0.052

Tube 2) comprises a pH 7.8 phosphate buffer solution. This can be sourced as a ready formulated buffer at £15 litre-1 from BDH. As each test uses a 10 ml solution, this therefore has a £0.15 cost per test. Costs can be significantly reduced if the buffer is made using the component salts:
(a) sodium dihydrogen orthophosphate 125mg per 10 ml solution
(b) disodium hydrogen orthophosphate 36 mg per 10 ml solution
This results in a raw material cost of £0.003 per test if the solutions are made in this way.

4.5 Kit production protocol: Kits were prepared at scale by Martin Lishman Limited for the 2006 harvest trials using the following protocol:
a) 500mg XTT was mixed with 61.7ml of methanol in a dry, sealed glass container.
Methanol was measured out using a 100ml glass measuring cylinder and placed in a container immediately due to its volatility. This was stirred with a magnetic stirrer constantly during kit preparation and the lid kept on at all times except when taking out solution.
b) Azlon bottles were lined up in plastic trays and the bottle lids removed. 0.1ml of XTT solution decanted into each bottle using a Multipipette. After dispensing, the bottles are left in the dark with the lids off until the XTT solution was completely dry (usually 1 hour at room temperature). When dry, the lids (pre-fitted with filters) were replaced on the Azlon bottles. The bottles were packed in batches of 50 upright into a black plastic light-proof bags and sealed then packed in to a cardboard box of appropriate size.
c) The 50 mMolar pH 7.8 potassium phosphate buffer was produced as follows: 250 ml of 1.0 mol Potassium Phosphate pH7.8 buffer was measured into a 500ml glass measuring cylinder and mixed with 4750 ml (4.75l) deionised water in an empty clean 5 litre container. 500ml of the resulting solution was decanted into 10 x 500ml plastic dispensing bottles.

ii) Colorimeter

The initial test methodology envisaged comparing the solution colour with a colour chart to provide the bruise index. In practice, however, the intensity of the solutions was not sufficient to provide a practical reading in the field. As a consequence it was decided that a colorimetric method would need to be developed. Initial development was undertaken with a Biochrom 8-3000-44 CO7500B colorimeter. This was quoted at around £324 per unit
After looking at the marketplace for colorimeters, at £140, a Hanna HI-93727 Colour of water meter was identified as a potential, lower cost alternative. The key issue however was that whilst the instrument operated at the correct wavelength (470 nm) for the bruising analysis, the instrument was designed for water analysis and was far too sensitive leading to overloading and error signals for all but the lowest bruising indices. After consultation with the manufacturer, it was agreed that the instrument could be modified to provide the appropriate sensitivity and to directly provide a bruise index reading from the instrument. Appendix C3 defines the required modifications to the Hanna 93727 “colour of water” instrument to permit scale readouts in bruise index units. Two Hanna units with reprogrammed EPROMS were despatched to Durham for comparison and validation against the Biochrom and laboratory instrumentation. Excellent correlation was achieved as detailed in Appendix C4. As a consequence, a further 8 units were ordered for distribution to the consortium for use in the harvesting trials in autumn 2006.

4.6 Kit Pricing: **Table 4.3** illustrates the cost improvements achieved through sourcing of materials and simplification of product construction. The sales format for the product is envisaged to be an initial purchase of a kit containing all the test equipment and sufficient consumables for 50 tests. Subsequent purchases would be of consumables for the individual tests.
The estimated costs for the key kit components are summarised in **Table 4.4**

The total cost for the kit as supplied for 50 tests is around £250-£300 with a consumable cost per test of around 40p. This equates to a target sale piece of c£500-£600 for a 50 test kit with a repeat purchase test cost of 80p to £1. The cost of assembly/manufacture of the kit in Canada by BioVectra was prohibitively expensive.

Table 4.3 Component prices and cost improvements through sourcing and simplification of product construction

Component (price per test for consumables)	2004	2006
XTT	£0.50	£0.07
Aslon bottle	£0.56	£0.21
Filter	£0.20	£0.006
Phosphate buffer	£0.15	£0.003
Impactor and cutter	£300	£55
Colorimeter	£324	£140

Table 4.4 Estimated costs for the key kit components

Corer and bruise	£55	
Colorimeter	£140	
Dispenser	£65	Probably less than this
Timer	£1.55	
XTT	£6.50	Per test
Aslon Bottle	£10.50	21 p per bottle
Filter	£0.60	0.6p each
Phosphate buffer	£1.50	

4.7 Commercial roll out strategy

The basic principles that have been adopted are as follows:

1. That there is to be no roll out until the partners agree that the kit meets its performance objectives in the field
2. That there is no commercial activity until this point is reached – except a holding response to enquiries
3. That Martin Lishman Limited's role is as manufacturer and distributor. The content and function of kit and interpretation of results by users (including instructions for use) would be dictated by the LINK partners that have trialled the kit.
4. Commercial roll out is currently on hold, pending a review of trials undertaken in 2007. This is expected to be concluded in a BPC coordinated meeting in February 2008.

Confirmation that the commercial objectives had been met would envisage having the kit ready for market in time for the next harvest. This would be accompanied by promotional activity including:

- ***press releases to potato specialist and general journals***
- ***a possible press launch by BPC***
- ***potential use of BPC mailings to growers***
- ***LINK partners to carry out mailings to their growers***
- ***information on BPC and partner websites***
- ***brochure for distribution to enquirers***
- ***future BPC events (eg.a Sutton Bridge storage event, BPC demo/show)***

Part 5 - Studies on the biochemical and physiological responses of tuber cells to impact

Objective 5 – Biochemistry of responses
Task 18 – Pectin and cell signals
Task 19 – cell wall structure and composition

5.1 Introduction

This section of the report deals with a lengthy series of biochemical experiments designed to further understand the basis of the bruise susceptibility – superoxide generation correlation reported previously. The results of this work have also been prepared as a paper entitled *"The Dissection of the Biphasic Oxidative Burst Response of Potato Tuber Cells to Abiotic Stress"* for submission to *Plant Physiology* or an equivalent journal (see also **Part 7** - **References**). It represents a follow-on paper from the original description of the relationship between superoxide radical generation and bruise susceptibility (Johnson and Croy, 2003). The full paper is provided as a pdf document in the Appendices. The following **Section 5** of the report contains the experimental sections, results and conclusions with additional relevant figures and materials. The references are contained within **Part 7 – References.**

5.2 Dissection of the biochemical responses

Scientific Background The processes whereby cells in potato tuber cortical tissue perceive a mechanical stimulus and respond with an apoplastic biphasic oxidative burst have been investigated at a number of levels using tissue bioassays. Following a shock wave passing through the tissue, the primary phase response involves the initial generation of superoxide radicals through the activation of a plasma membrane NADPH-oxidase. The initial radical generation then declines before a secondary phase generation is elicited by the scission products of cell wall components promoted by radicals arising from the primary phase. Through the use of a series of chemical inhibitors and modifiers the activation of these responses has been shown to entail cell signalling events involving a protein kinase and phosphatase system and ion fluxes which involve protons, calcium and potassium ions. The elicitors which initiate the second phase oxidative burst are derived by radical scission of various cell wall pectic polysaccharides and are a heterogeneous mixture of at least two size fractions comprising of molecules > and < 5kDa. The kinetics of the elicitation response has been elucidated.

Two models for the generation of apoplastic AOS have been proposed in plants. The first is a superoxide generating system utilising a transmembrane NADPH-oxidase as seen in mammalian neutrophils (Doke, 1995). In this system the presence of a transmembrane NADPH-oxidase was demonstrated with free radical generation depending on intracellular signalling cascades involving GTP-binding proteins, ion channels, protein kinases and phosphatases, phospholipase A and C, as well as cAMP. Previously we suggested that this system operated in potato tuber cells subjected to mechanical stress (Johnson *et al.,* 2003). The alternative system for AOS generation is one which has no parallels in animals. In this system greater emphasis is placed upon ion fluxes through membrane channels. Membrane depolarization is a well characterized response immediately prior to the oxidative burst and typically involves Ca^{2+} and H^{+} ion influx from the extracellular matrix (ECM) into the cytosol with a corresponding K^{+} and Cl^{-} efflux into the ECM. These ion fluxes lead to a transient alkalinisation of the apoplastic fluid, which is demonstrable in cell suspension cultures treated with elicitors (Abdullah, 1998). This change in pH is proposed to activate a pH-dependent cell wall peroxidase leading to accumulation of apoplastic hydrogen peroxide. (Bolwell *et al.*, 1995). The oxidative burst in tuber cortical cells observed following abiotic mechanical stress, is associated with a number of other phenomena including protein oxidative modification and pigment generation (Johnson *et al.*, 2003). Quantification of levels of superoxide generated following impact were found to be directly correlated with the potatoes propensity to form blue-black melanin–based pigments known as 'blackspot bruising', an oxidative reaction initiated by the enzyme polyphenol oxidase acting on its tyrosine substrate (Corsini *et al*., 1992; Stevens and Davelaar, 1996, 1997; Friedman, 1997). Tuber bruising is a significant agricultural problem during harvesting, transport and processing causing serious financial losses (Van es Rastovski, 1987; Kleinschmidt and Thornton, 1991; Potato Marketing Board, 1994).

Potato tubers provide a convenient tool for studying the oxidative burst because the superoxide generation is highly reproducible and easily quantified. Potatoes are also available in a wide range of genetic lines which display different susceptibilities to 'blackspot bruising' and due to the close relationship between this and superoxide generation, material is available for study in which the oxidative burst is highly active, and others where the oxidative response is almost non-existent. While the underlying basis for this relationship has still to be elucidated, a thorough understanding of mechanical stress-induced oxidative burst in potatoes could realise substantial benefits to the agricultural industry.

Previous work proposed a model for potato tuber cells in which mechanical impact was perceived by tuber cells, possibly through mechanoreceptors, ion channel proteins or membrane perturbation, which instigated an initial phase of superoxide generation through signaling cascades which activated various enzymic processes including the NADPH-oxidase complex. The superoxide generated is apoplastic in origin hence the basis for the *Blackspot Detect* assay, and therefore this may trigger free radical scission of cell wall polysaccharides yielding biologically active fragments. These fragments can induce a *de novo* oxidative burst in potato tuber cells where no previous stress had been imposed and thus these are taking part in a 'self-elicited' amplification of superoxide generation through a slightly different activation route. Inhibition studies have now shown that superoxide generation is primarily through a plasma membrane NADPH-oxidase complex.

Materials and Methods

Potato Tuber Materials Potato varieties *cv* Cara and *cv* Russet Burbank were grown specifically under monitored field conditions, harvested manually and stored in the dark at 4°C to delay sprouting. Bruise susceptibility of tuber samples were estimated according to Johnson *et al.* (2003).

Bioassays for Superoxide Generation by Tuber Cells

Assays were based on the methods described by Johnson *et al.* (2003). Standard cores of tuber cortical tissue (4.5mm x 10mm) each weighing ~330mg were excised from test tubers using a coring device. Test tubers were impacted with a specially designed spring-loaded piston (6 mm head diameter) calibrated to deliver a precise impact energy of 0.7J (Croy *et al.* 1998), and then incubated at 20^{o}C for appropriate periods before a core was excised at the point of impact. Cores were washed briefly in Milli Q water, blotted and incubated at 20°C for 20 min in 200μl of 0.12 mM XTT in 50 mM potassium phosphate buffer, pH 7.8 (Able *et al.*, 1998). Absorbance change at 450nm was converted to nanomoles of superoxide generated.g^{-1}.min^{-1} using the molar extinction coefficient for the XTT formazan product of 23,600 m^{-1}.cm^{-1} (Sutherland and Learmonth, 1997). For the investigation of short term effects, absorbance readings were taken at 1 minute intervals after the temporary removal of the cores from the assay solution. Elicitor preparations were assayed by pre-incubating unimpacted cores in serial dilutions of elicitor for 10 minutes, washing and then incubating in XTT for 20 minutes as before. The effects of inhibitors and activators were investigated by pre-exposing cores to serial dilutions of test solutions in 50mM phosphate buffer pH 7.8, followed by washing where indicated, and assaying with XTT. The effects of transient alterations to the intracellular pH were investigated by placing cores in elicitor plus different concentrations (5mM-50mM) of ammonium chloride or potassium acetate in 50mM phosphate buffer pH 7.8 for prescribed times (1-10, 20 and 30 minutes), then the cores were removed washed and assayed for superoxide generation.

Cell wall and Starch Grain Isolation and Purification

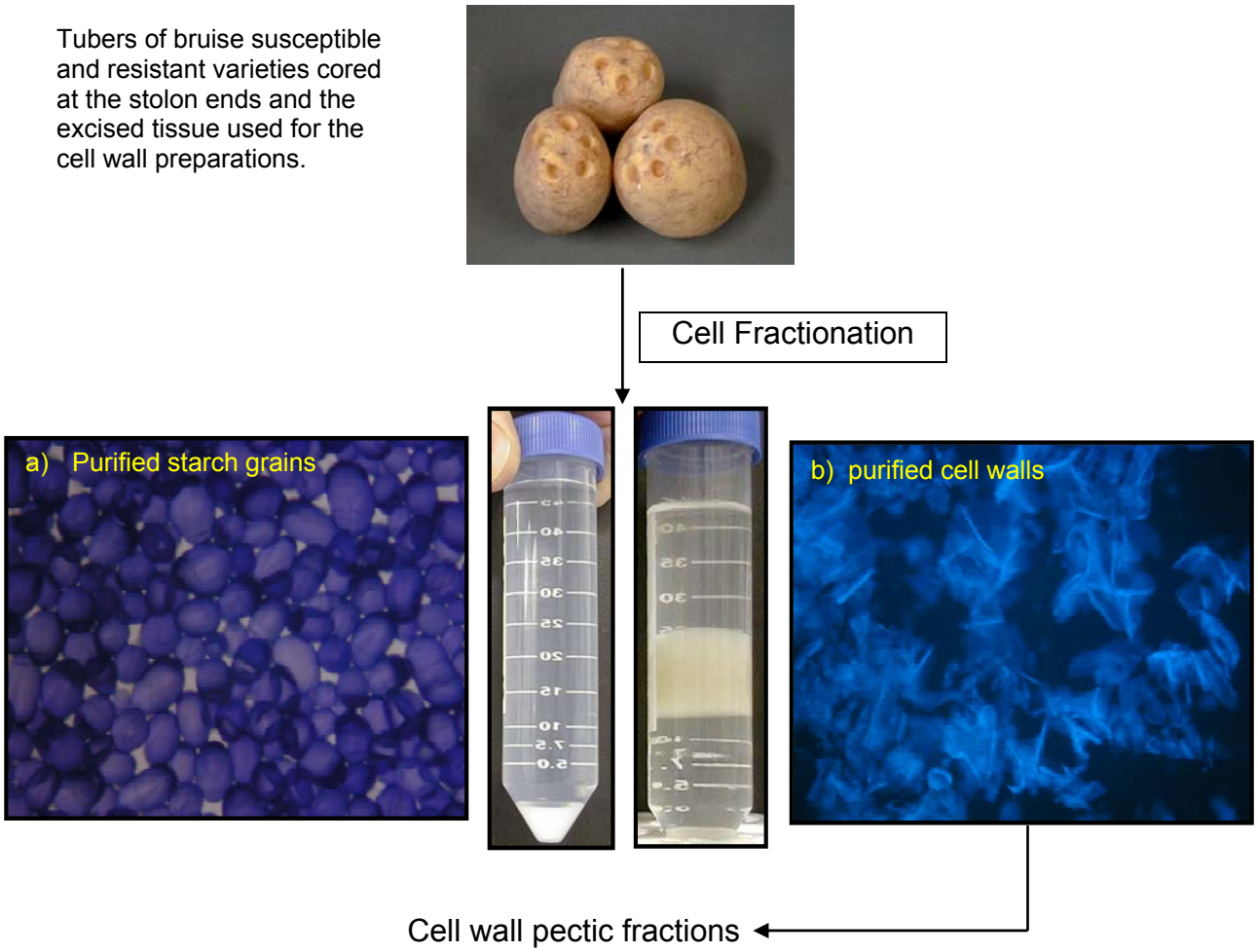
Tuber cell walls were prepared by an adaptation of the methods of Johnson *et al.* (2003) and Sorensen *et al.* (2000). Unless indicated all steps were carried out at 4^{o}C. 60g of tissue cores were excised from the stolon cortex region of selected tubers and initially homogenized using a handheld blender (Braun 600 watt) in 100ml of 2mM dithiothreitol (DTT) for 3 x 10 sec and then filtered through 4 layers of muslin. The residue retained in the muslin was re-homogenised twice more in 50ml aliquots of 2mM DTT. The filtrates were retained for starch grain isolation. The residue in 50ml of 2mM DTT was then subjected to three rounds of homogenizations using a Polytron PT10-35 (Kinematica) fitted with a 13mm aggregate and operated at speed setting 6-7 for 3 x 10 sec followed by centrifugation at 3000g x 5 min (Jouan BR4, S40 rotor). The buff-coloured cell wall material was washed free from any white pellet of residual starch. The final pellet was resuspended in 50ml of water, frozen as beads (~50μl) in liquid nitrogen, comminuted to a frozen powder in a blender (Yellowline, A10) and then allowed to thaw at room temperature. This step was repeated once more. The cell walls were pelleted by centrifugation as before (Jouan BR4, S40 rotor) and the pellet resupended in 50ml of water. Residual starch grains were gelatinized by heating at 80^{o}C for 30 min, cooled

and the cell wall material recovered from gelled starch by centrifugation at 3000g x 10min. Finally the cell wall material was purified on a stepped gradient comprising of 10ml of 100% Percoll (Sigma P1644-1L) overlaid on a cushion of 10ml of 90% (w/v) aqueous sucrose and centrifuged at 2000rpm x 5 min. The cell walls formed a thick buff coloured band within the Percoll layer and were removed, diluted to 50ml with water and recovered by centrifugation as before 3000g x 10min. The cell walls were washed 3 times and freeze dried. This material was used for further purification by the methods of Sorensen *et al*. (2000), except only one round of the destarching procedure was used. The final preparation was freeze dried or used directly for the isolation of pectic oligo and polysaccharides. Starch grains were isolated by sedimentation from the filtrates of the initial homogenates followed by six rounds of resuspension in water and centrifugation and finally freeze dried. The purified cell wall and starch fractions were examined by light microscopy after staining with Calcofluor (0.1%) and Lugols iodine.

Isolation of Cell Wall Pectic Polysaccharide Extracts

Pectic extracts were made from the purified cell wall materials (**figure 5.1**) according to the methods described by Sorensen *et al.* (2000) and Johnson *et al.* (2003) except 25mg dry weight of the purified cell wall material were extracted, firstly with EPG/PME enzymes (fraction 1), then with carbonate, borohydride and EPG (fraction 2) and finally the insoluble cell wall residue (fraction 3) was recovered. These fractions were assayed alongside the starting cell wall material for the presence of elicitors before and after treatment with superoxide radicals.

Figure 5.1 - Tuber cellular fractionation yielding purified a) starch grains and b) cell walls.



Superoxide Scission of Cell Wall Fractions

Superoxide was generated chemically using a phenazine methosulfate (PMS) / NADH system described by Johnson *et al.* (2003) and in **Part 1.8**. Superoxide production using this system is exhausted by 25 minutes. Crude and purified cell wall preparations, starch grains and isolated cell wall fractions were treated as

suspensions (10mg/ml) or as solutions, with an excess of superoxide for 30 minutes before being tested for elicitor content in bioassays.

Sizing Elicitor Fractions by Ultrafiltration

The size range of the elicitors produced by superoxide scission was estimated by fractionating superoxide-treated cell wall material using ultrafiltration (*Vivaspin 15R, Hydrasart filter, MWCO 5000*). Purified cell wall material was exposed to an excess of superoxide and scission allowed to proceed to completion. 15-20ml of elicitor in 100mM potassium phosphate buffer pH 7.8 were placed in the upper chamber and the device centrifuged at 3000g (*Jouan BR4, S40 rotor*) at 4^{o}C for 30-40 minutes until about 10ml of filtrate was recovered in the lower chamber. The filtrate was removed and 10ml of fresh buffer were added to the upper chamber and the centrifugation repeated. The process was repeated a further 6 times and the separate filtrates and high mass fraction retained in the upper chamber were tested for elicitor content.

Statistical Analyses

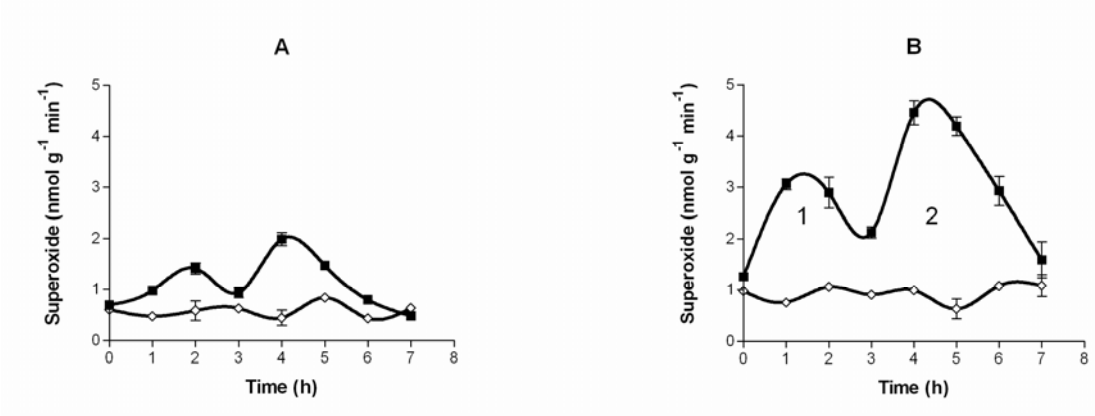
Statistical analyses were performed on all replicated data sets using *Prism* software (*v3.02, Graphpad Software, San Diego*). Sample standard deviation were calculated from replicate results and used to add the error bars to all figures. All data used for graphs were from independent experiments replicated between two and six times as indicated.

Results

The Biphasic Oxidative Burst

Superoxide generation profiles following a standard mechanical impact of 0.7 joule were monitored to reaffirm the difference in mechanical stress susceptibility between the two varieties tested and to reconfirm the biphasic superoxide generation. Cara (figure 1A), a resistant variety, shows very little superoxide generation over the 7 hours following impact, whereas Russet Burbank (figure 1B) is a susceptible variety and shows the characteristic biphasic profile of superoxide generation following the impact. In devising a model for the tuber cell response system we have investigated the two components of the biphasic response as indicated in Figure 1B as phase 1, the primary response arising from mechanical stress and phase 2, the secondary response, arising from the influence of cell wall derived elicitors.

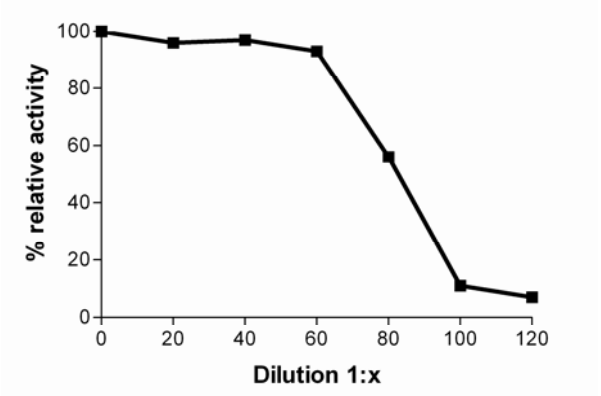
Figure 5.2 - **Biphasic generation of superoxide radicals by tuber cortical cells** from A) Cara and B) Russet Burbank varieties in impacted (■—■) or non-impacted (◇—◇) tissue cores. The primary phase (1) peaks at 1-2h after impact the secondary phase (2) peaks 4-5h after impact as shown in Figure 1B. Experiments were carried out in triplicate and the error bars represent sample standard deviations.



Activity of Elicitor Preparations

Elicitors were prepared by the action of superoxide radicals on purified tuber cell walls as described previously (Johnson *et al.* 2003). It was clear that tuber whole cell walls treated with superoxide radicals led to production of soluble extracts which elicited an apoplastic oxidative burst in non-impacted tuber cortical cells. In order to estimate the yield of active fragments in independent preparations, dilution series were set up and assayed for elicitor activity (figure 2). At a dilution of 1:60 the activity of the most active preparation was still near maximum and this dilution was subsequently adopted as the standard working dilution. For comparisons between elicitor preparations, a 1:60 dilution of a standard cell wall preparation processed under identical conditions was included in all sets of assays. Results were expressed relative to this activity.

Figure 5.3 - **Quantitation of active elicitor preparation by dilution.** A 1:60 dilution is the highest dilution which still achieves the maximum activity of this elicitor preparation. Data shown are the results from three independent assays. Error bars represent sample standard deviations.

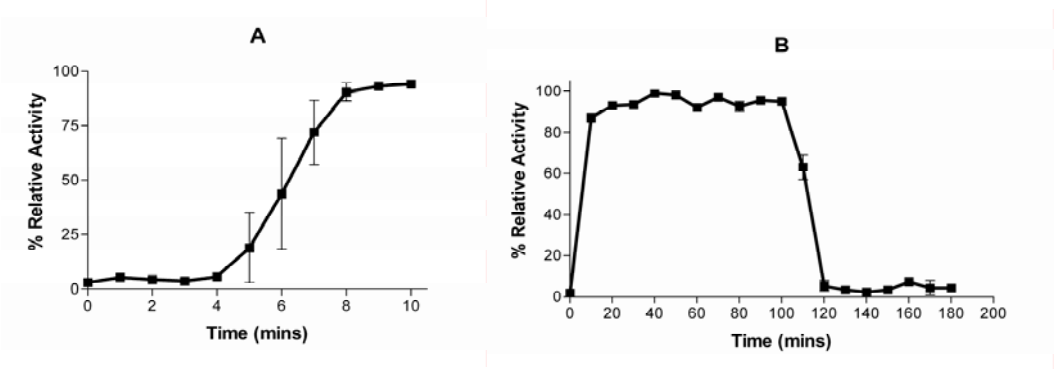


Kinetics of the Onset and Duration of Elicitor-induced Superoxide Generation

By exposing tissue cores to elicitor for different times and then immediately monitoring the generation of superoxide it was possible to elucidate the kinetics of the system. Figure 3A demonstrated that superoxide generation commences 4-6 minutes after exposure of the cells to elicitor solution. Activation is rapid and maximal generation of superoxide was reached within 8 minutes following exposure to the elicitor.

Figure 3B shows that the superoxide generation following elicitor exposure lasts for around 100 minutes before decreasing to near zero. This implies that once bound the elicitor-receptor complex is stable for this period during which time the events leading to radical generation are fully activated. Alternatively it is possible that this result reflects the period of sustained activation of the signalling components. The rapid decline of radical generation indicates precisely controlled events, probably manifested by a swift and synchronised activation produced by the initial elicitor binding and the rapid activation.

Figure 5.4 - Kinetics of elicitor-induced activation
A – activation time: tissue cores were exposed for between 0 (control) and 10 minute periods to a standard elicitor preparation, washed briefly in water and then assayed immediately for generation of radicals. The data shown are the results from three independent assays.
B – duration of elicitor activation – tissue cores were exposed to elicitor for 10 minutes, then these were transferred to potassium phosphate buffer for periods of up to 180 minutes (0 to 200 minutes following exposure to elicitor. The cores were then assayed for radical generation. The data shown are the results from three independent assays. All error bars represent sample standard deviations.



Phase 1 and 2 Responses

The effects of a number of inhibitors and activators on the level of oxidative burst generated by mechanical impact (phase 1 response) and induced by cell wall elicitors (phase 2 response) were investigated. Cyanide was used to eliminate enzymes which could influence superoxide levels by reverse catalysis of H_2O_2 (originating from exocellular peroxidase) by SOD or its removal by endogenous enzymes – SOD and PPO. Cyanide effectively inhibits exocellular peroxidase (Bolwell *et al.* 2002), superoxide dismutase (Fidalgo *et al.*

Figure 5.5 A-D Influence of inhibitors and activators on phase 1 superoxide generation
Impacted cores were pre-incubated with cyanide, A23187, PMA or cycloheximide for 10 minutes, washed and then assayed for superoxide generation.
A - Cyanide control (∗—∗), 0.1mM (■—■), 0.5mM (▲—▲), 1mM (▼—▼).
B - A23187 control (∗—∗), 0.1µM (◆—◆), 0.5µM (●—●), 1µM (□—□).
C - PMA control (∗—∗), 10nM (△—△), 50nM (▽—▽), 100nM (◇—◇).
D - Cycloheximide control (∗—∗), 50µg/ml (○—○),100µg/ml (×—×), 250µg/ml (+—+).
All data are the result of triplicate assays and the error bars represent sample standard deviations.

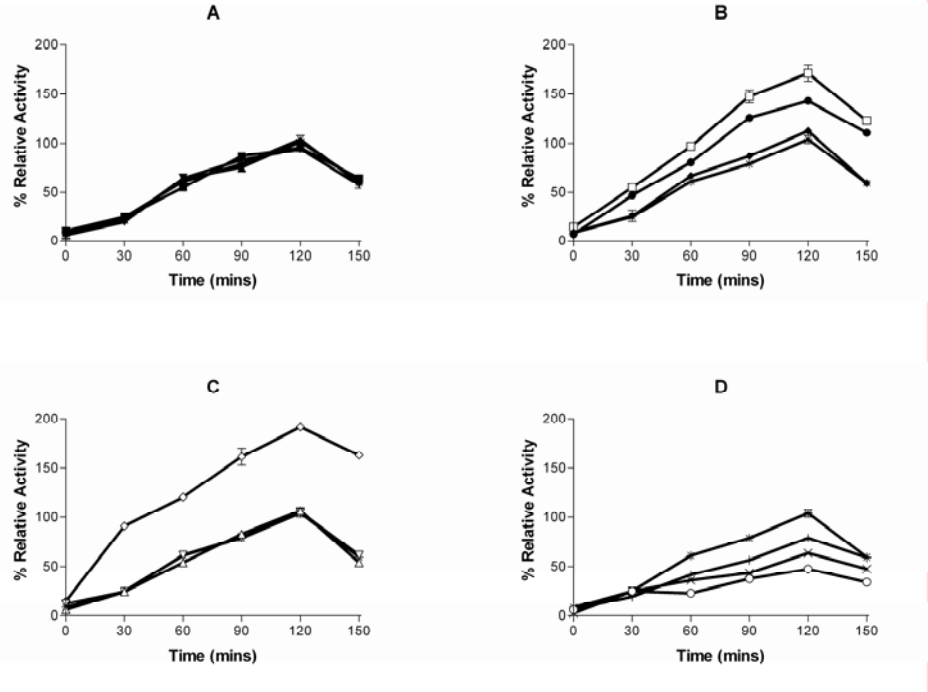
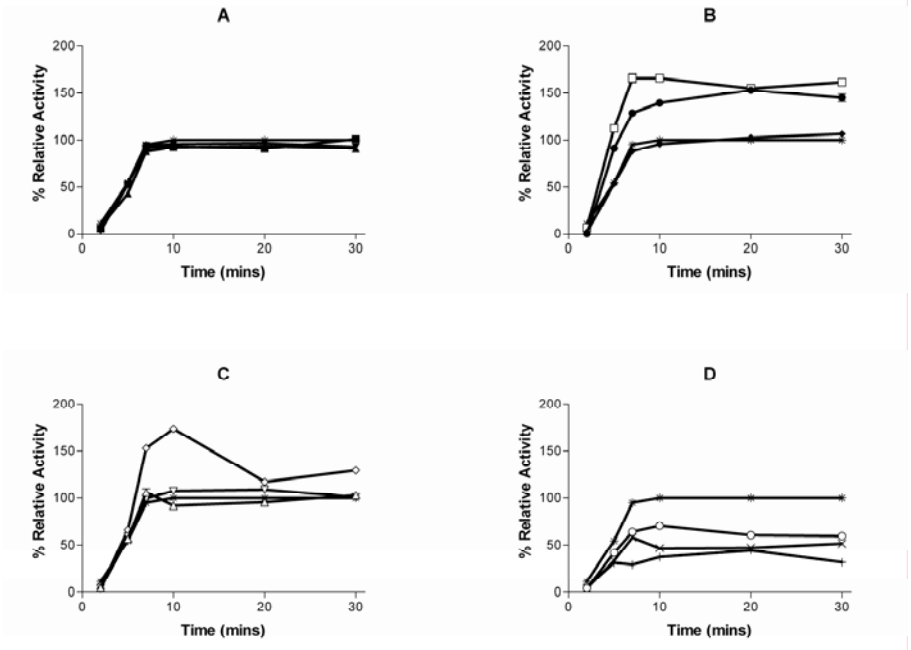


Figure 5.6 A-D Influence of inhibitors and activators on phase 2 superoxide generation
Non-impacted cores were treated with elicitor preparation and a range of concentrations of the inhibitors or activators, washed and then assayed for superoxide generation.
A - Cyanide control (∗—∗), 0.1mM (■—■), 0.5mM (▲—▲), 1mM (▼—▼).
B - A23187 control (∗—∗), 0.1µM (◆—◆), 0.5µM (●—●), 1µM (□—□).
C - PMA control (∗—∗), 10nM (△—△), 50nM (▽—▽), 100nM (◇—◇).
D - Cycloheximide control (∗—∗), 50µg/ml (○—○),100µg/ml (×—×), 250µg/ml (+—+).
All data are the result of triplicate assays and the error bars represent sample standard deviations.

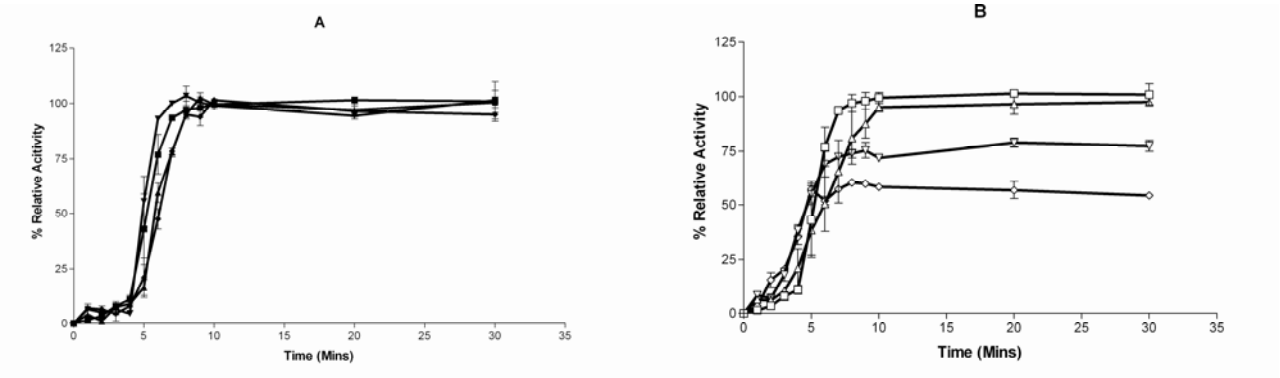


In contrast, the calcium iononophore A23187, which promotes the uptake of extracellular calcium, strongly increased superoxide generation at concentrations of 0.5µM and above in a dose-dependent manner. This suggests a significant role for intracellular calcium influx in the phase I response. PMA, an activator of protein kinase C, also showed a highly stimulatory effect albeit only at a threshold concentration of 100nM but thereby indicating the activation of a protein modification cascade in the signalling system associated with the phase I response. The translation inhibitor cycloheximide showed a dose specific inhibitory effect, however the degree of inhibition was no more than 50% even at the highest level of inhibitor tested (250µg/ml), suggesting only a partial role for *de novo* protein synthesis.

The same chemical treatments were applied to the phase 2 generation of superoxide (i.e. that due to self-elicitation of tuber cells by oligosaccharides). It was noted that cyanide again showed no inhibitory effect over the range of concentrations tested, indicating that superoxide generation in this secondary phase is also due to the plasma membrane NADPH-oxidase complex unaffected by other oxidative enzymes. As in response 1, the involvement of extracellular calcium was demonstrated by the strongly stimulatory effect of 0.5µm ionophore A23187. Interestingly PMA in phase 2 appeared to have only a transient effect with a strong promotion of superoxide generation within the first 10 minutes and only at the highest level of PMA (100nM). This hyper-stimulation had declined by 20 minutes. Cycloheximide inhibition of superoxide generation in this phase was manifested 17 minutes after first exposure to the elicitor and in a dose dependent manner.

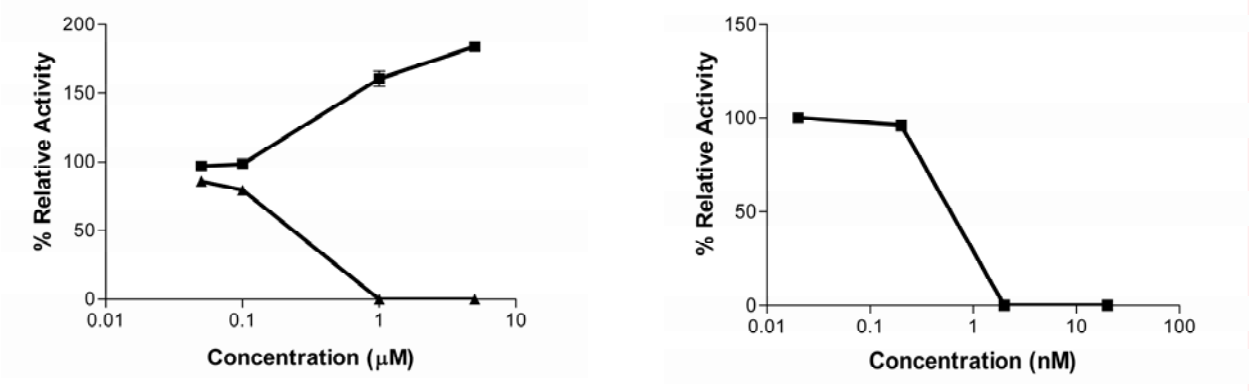
Plant oxidative burst systems involve the establishment of a pH gradient across the plasma membrane involving H^+, Ca^+ and K^+ ion fluxes (Doke and Miura, 1995, Abdullah, 1998). Transient alkalinisation of the apoplast is required to activate the exocellular peroxidase AOS generating system proposed by Bolwell *et al. (*2002). Therefore we investigated the effects of altered proton gradients across the plasma membrane on phase 2 radical generation by exposing tuber cells to transient changes in their intracellular pH. Increasing the intracellular pH with ammonium chloride showed no influence on radical generation throughout the range of concentrations tested. In contrast, decreasing the intracellular pH by addition of potassium acetate, showed a marked lowering of radical generation and in a dose dependent manner (Fig 6 A-B).

Figure 5.7 A-B Effects of manipulating intracellular pH on phase 2 superoxide generation
Cores were pre-incubated with elicitor in the presence of A) ammonium chloride or B) potassium acetate solutions buffered to pH 7.8, for periods of up to 30 minutes, washed and then assayed for superoxide generation.
A – increasing intracellular pH by addition of extracellular ammonium ions. Control (■—■), 5mM ammonium chloride (▲—▲), 10mM ammonium chloride (▼—▼), 50mM ammonium chloride (◆—◆).
B – decreasing intracellular pH by addition of extracellular acetate ions. Control (□—□), 5mM potassium acetate (△—△), 10mM potassium acetate (▽—▽), 50mM potassium acetate (◇—◇). All data are the result of triplicate assays and the error bars represent sample standard deviations.



At concentrations above 1µM the potassium ionophore valinomycin strongly promoted superoxide synthesis following elicitor exposure, indicating a significant role for this ion in this phase (Fig.7A). In contrast staurosporine, a potent inhibitor of protein kinases, completely inhibited the response at concentrations of 1µM and above. This agrees with the previous results showing activation of the response by PMA and further implicates protein kinases in the pathway controlling activation of superoxide generation following elicitation.

Figure 5.8 - Analyses of the second phase response. Responses assessed with varying concentrations of A) ■—■ valinomycin (0.05µM – 5µM), a potassium ionophore, ▲—▲ staurosporine (0.05µM – 5µM), a protein kinase inhibitor and B) ■—■ okadaic acid (0.02nM – 20nM), a protein phosphatase inhibitor. Cores were pre-exposed to elicitor in the presence of activator or inhibitor for 10 minutes, washed and then assayed for superoxide generation in the standard 20 min assay. All data are the result of triplicate assays and the error bars represent sample standard deviations.



In a complementary set of experiments okadaic acid, an inhibitor of protein phosphatases, was shown to completely inhibit the second phase response at concentrations of 2nM and above. This provides further evidence for the involvement of protein activation / deactivation signalling events in the phase 2 response (Fig. 7B).

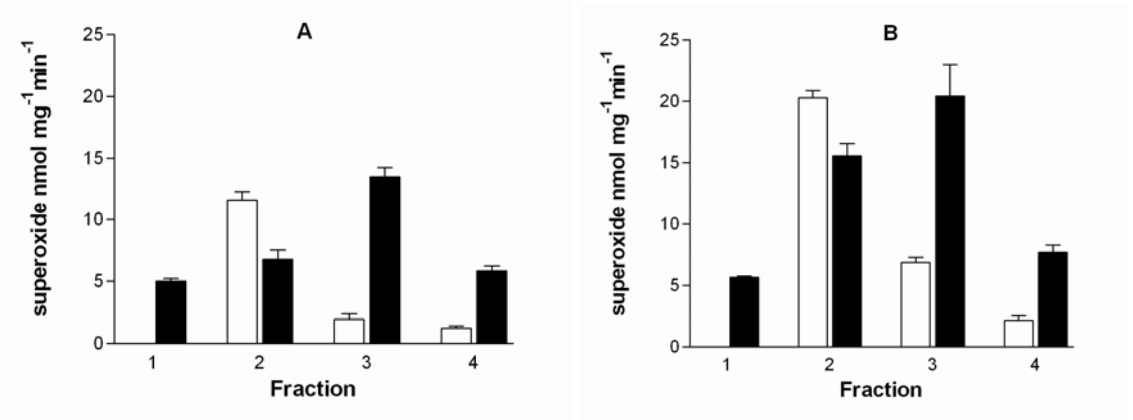
Elicitors Derived from Isolated Pectic Fractions of the Cell Walls

Previous work with this system suggested that elicitors capable of activating radical generation in tuber cells were derived from cell wall components and most probably from pectic polysaccharides (Johnson *et al.,* 2003). To characterise the source of the elicitor more definitively pectic poly- and oligo-saccharides were isolated from highly purified cell walls of tuber cortex tissue from both test varieties. The fractions isolated were 1 – endopolygalacturonase (EPG) enzyme extract; 2 – carbonate/borohydride extract and 3 - the final cell wall residue, corresponding to similar fractions isolated by Sorensen *et al.* (2000). The three purified fractions were tested for elicitor activity along with samples of the starting material, before and after treatment with superoxide radicals. The results are presented in Table I and Figures 8A and B and confirm the pectic polysaccharides as the source of the elicitors inducing the second phase oxidative burst. Interestingly the yield of elicitor activity was much higher in the isolated fractions than that released by superoxide treatment of cell walls alone. A quantitative difference was noted between the pectin-derived elicitors from the two varieties.

Table 5.1 - Mean levels of radical generation from pectic fractions isolated from Cara and RB varieties. SO generated is measured in nmoles $SO.g^{-1}.min^{-1}$.

fraction	Cara untreated	Cara SO treated	Russet untreated	Russet SO treated
1. Purified CW	0.0	5.0	0.0	5.7
2. EPG enzyme extract	11.6	6.8	20.3	15.6
3. carbonate/ borohydride extract	2.0	13.5	6.9	20.4
4. CW residue	1.3	5.8	2.2	7.7
Total activity	14.8	26.1	29.3	43.7

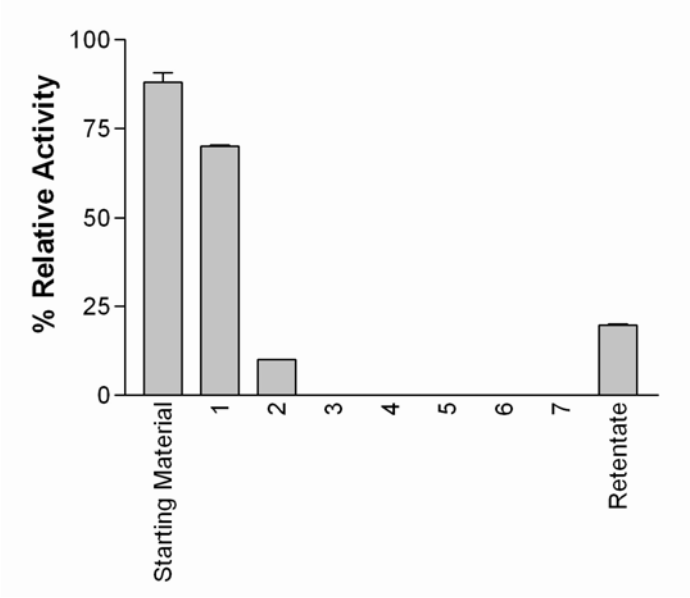
Figure 5.9 A – B Relative activities of elicitor preparations from purified pectic fractions. 1 – total purified cell walls (starting material), 2 – pectic fraction I, 3 – pectic fraction II, 4 – cell wall residue Filled bars – elicitor activities in untreated preparations; open bars – elicitor activities in preparations after treatment with superoxide radicals. All data are the result of triplicate assays and the error bars represent sample standard deviations.



Size of active elicitor

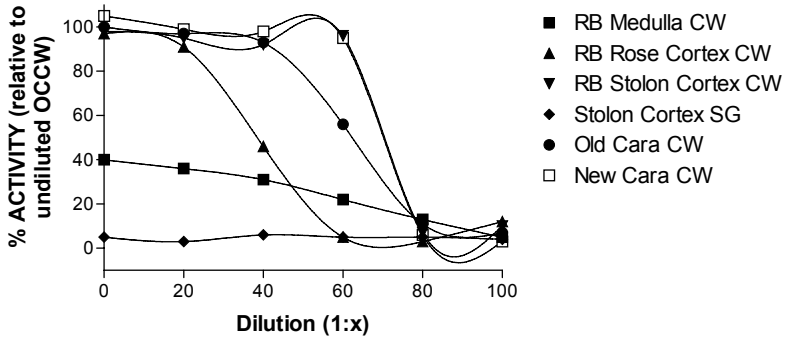
By using a 5000MWCO filter to separate high and low mass molecules it was possible to identify two distinct biologically active size fractions. Around 80% of the total activity was found in the low molecular mass material (<5kDa) but even after 7 washes almost 20% of elicitor activity remained in the higher molecular mass (>5kDa) fraction. This suggests the presence of at least two populations of biologically active molecules.

Figure 5.10 - Size fractionation of elicitor preparation from whole cell walls. Results shown are the % relative activity of 1-7 ultrafiltrates - low mass (<5kDa) fractions and high mass (>5kDa) retentate fraction retained by the filter. All data are the result of triplicate assays and the error bars represent sample standard deviations.



Elicitors derived from different tuber tissues: Tubers comprise of different tissues. It is well established that there are spatial differences in the properties of tissues from different ends of tubers. For example the stolon end which was attached to the parent potato plant, is more susceptible to bruising than the rose end. Potentially this could mean that the cell walls from tissues at one end of the tuber differ in their composition from those at the other end. Based on the findings described above a comparative experiment was conducted using tissues derived from different parts of tubers from a resistant potato variety (*Russet Burbank*). Cell wall fractions were isolated from the rose end cortex, the stolon end cortex, and medullary tissues as described previously and freeze dried. The levels of elicitor produced by superoxide radical scission of each fraction was then estimated by assaying the products by serial dilutions in the tissue bioassay for SO generation. The activities were related back to a highly active sample derived from *Cara* cell walls and expressed as a %. The results are presented in **Figure 5.11**. Stolon cortex tissue showed the highest levels of elicitor in both *Russet* and *Cara*, while the rose end produced only about 50% of the level of elicitor. The medulla produced very little elicitor and the starch grains (control) none.

Figure 5.11 - Elicitors derived from different tuber tissues



Discussion: In a previous paper we reported a genetic predisposition of particular potato varieties to mechanical stress susceptibility as measured by the localised deposition of melanin pigments in tuber subdermal tissues - 'blackspot bruising'. This and other studies have shown the genetic trend may be influenced by environmental conditions during different growing seasons (O'Leary and Iritani, 1969; Hudson, 1975; Skrobacki *et al*., 1989; Dixon, 1992; Sieczka and Thornton, 1993; British Potato Council, 2000). Varieties susceptible to mechanical stress were revealed by a localised generation of high levels of superoxide radicals and a high level of bruising, whereas 'resistant' tubers showed little or no superoxide generation and consequently low levels of bruising (figure 1A and B).

Overall we demonstrated a tight relationship between degree of mechanical susceptibility and level of radical generation providing a convenient tool for studying the mechanically induced oxidative burst. We further showed that the profile of superoxide generation in a susceptible variety displayed a characteristic biphasic pattern of generation, the first peak (around 2 hours post-impact) we described as a response to the impact, whereas the second peak (around 4 hours post-impact) we suggested was the result of oxidative scission of the cell wall by superoxide radicals generated during the first phase, yielding biologically active oligosaccharide fragments, as suggested by Fry (1998).

Two potato tuber varieties were selected for this study: Cara, a resistant variety, and Russet Burbank, a susceptible variety. Figures 1A and 1B demonstrate the profound difference in superoxide release at the site of mechanical impact in these two varieties. Our earlier work showed that both Cara and Russet Burbank respond to a cell free extract from Russet Burbank by generating superoxide in a reproducible oxidative burst. To produce the active fragments *in vitro* we artificially generated free radicals using a PMS-NADH system in the presence of purified potato cell walls. The resulting biologically active preparation was found to be near maximally active at a concentration of 1:60 (Fig 2). In this artificial system the level of superoxide generated per minute is comparable to that seen in susceptible potato tubers, suggesting that *in vivo* the biologically active fragment is likely to be present in considerable excess.

The initial impact-induced burst of superoxide *in vivo* is followed by a depression in superoxide generation 3 hours post impact and then by a second, elicitor-induced burst of superoxide at 4 hours following impact. We tested this second phase to ascertain how long potato cells take to respond to elicitor exposure. Surprisingly, activation only took 6-8 minutes, with all experimental replicates reaching maximum superoxide generation within 10 minutes. The rapid activation of the oxidative burst following immersion of tissue cores in a solution of elicitor indicates a high level of synchronisation. This implies that only the outer cell layers of the tissue cores come into contact with the elicitor solution and participate in the response. This substantiates our observations on the level of response by cores which have been bisected several times which showed that superoxide generation increased in proportion to the surface area of tissue rather than its volume (data not presented). This means that much fewer cells contribute to the observed response making the elevated radical generation even more striking. The rapid decline in radical generation after 100 minutes is most likely a further manifestation of this synchronisation but also indicates a highly controlled series of activation and deactivation events. In this respect factors such as elicitor dissociation, finite duration of the activation of signalling components and depletion of intracellular substrates or other components are worth consideration as causal factors. *In vitro*, after exposure to elicitor, superoxide was generated for a duration of 100 minutes which is in accord with the observed duration of the second phase *in vivo*. In contrast *in vivo* there is a lag period of 60-120 minutes before the second phase reaches its maximum (Fig 1B). We propose that the disparity between the *in vitro* measurements and the *in vivo* observations occurs because the phase 1 generation of superoxide takes place over a sustained time period (unlike the artificial system where generation is almost instantaneous) leading to a progressive accumulation of bioactive fragments. This gradual build up of fragments, coupled with relatively slow diffusion rates in the apoplast, accounts for the relatively slow onset of the second phase of superoxide generation, which we propose should be envisaged as overlapping that of phase 1, i.e. as phase 1 generation is subsiding then phase 2 generation is increasing, leading to a double peaked profile of generation.

We have sought to elucidate the nature of the events leading to superoxide generation in these two phases by exposing tuber cells to various chemical inhibitors or modifiers either following impact or elicitor treatment. Firstly, by inhibiting certain enzymes using cyanide, which might influence levels of superoxide by generating or removing this radical, it is probable that the primary source is NADPH-oxidase which is in accord with our earlier findings (Johnson *et al.,* 2003).

To further investigate the involvement of ion channels a series of experiments were performed. The potassium ionophore valinomycin showed a strong promotion of superoxide generation in response 2 indicating that after oligosaccharide elicitor binding has taken place depolarisation of the cells occurs in which potassium ions are pumped out of the cells in exchange for protons. The ionophore could facilitate this exchange by increasing the available intracellular concentration of potassium and would explain the significant enhancement of the oxidative burst. Similar observations have been reported by Minibayeva *et al.* (1997) and Kupper *et al.* (2001) which also suggest that potassium ion efflux plays an important role in the plant oxidative burst. Interestingly potassium as a nutritional component has been implicated for many years in agronomic strategies to control potato bruising especially in sandy or acidic soils. (Monday *et al.* 1967, Mulder 1956). This has been variously reported to be due to effects on polyphenoloxidase, tyrosine levels and wide-ranging fundamental biochemical effects such as on photosynthesis and metabolism however this is the first evidence to implicate potassium ions in a specific biochemical phenomenon directly related to blackspot bruising.

The primary phase is also known to act through ion channel depolarisation suggesting both responses may share commonality of signalling components. Calcium ionophore A23187 strongly promoted superoxide generation in both response 1 and response 2, a result not entirely unexpected given the well documented evidence for this ion as a signalling intermediate in a wide range of systems (Doke and Miura, 1995). The implication of this is that specific signalling cascades e.g. protein kinases, may be activated as a response to the facilitated calcium ions influx. PMA highly stimulated the superoxide generation, whereas staurosporine completely inhibited it which strongly implicates protein kinases as signalling intermediates in both response 1 and response 2. Results with cycloheximide suggested the requirement for key protein factors for activation or response and occurring in both responses. The short-term inhibition in phase 2 may be indicative that ongoing translation of some components was required for continued activation and radical generation. The level of inhibition was never 100% even at the highest level tested.

Transient acidification of the cytosol caused a significant and dose dependent inhibitory effect upon the elicitor-activated superoxide generation. We interpret this to be due to ion channel changes, which have

been proposed in this system by Laerke *et al.* (2000), and implies that proton influx takes place in response to elicitor binding but is repressed with elevated intracellular proton concentrations. Surprisingly, transient alkalinisation of the cytosol caused little or no effect on superoxide generation where an enhancement might have been expected. This may be attributable to the external basic cellular conditions limiting proton uptake.

Elicitors are Derived Primarily from the Pectic Components of the Cell Walls

The majority of the extractable pectic poly- and oligo-saccharides can be isolated from purified tuber cell walls based on EPG and PME enzyme solubilisation followed by cold extraction with sodium carbonate / borohydride as described by Sorensen *et al*. (2000). The levels of elicitor present in these fractions and in the pectin-depleted cell wall residues before and after superoxide treatment were measured by tuber core bioassays for both potato varieties. It was immediately obvious that the majority of the elicitor molecules were derived primarily from the two isolated soluble pectic fractions in both varieties with a small amount coming from material left in the cell wall residues. All three preparations exhibited elicitor activity without additional superoxide treatment. Our previous work (Johnson *et al*., 2003) showed that pectinase enzymes such as EPG were capable of generating active elicitor fractions from purified cell walls, so it was not unexpected that the pectic fractions solubilised by such enzymes in the present study displayed endogenous elicitor activity without further treatment (Table 1, Figure 8A and B). The relative distributions of elicitors between the three fractions in both varieties were very similar though there were clear quantitative differences in the yields with Russet Burbank showing significantly higher levels of elicitor in all three pectic fractions tested. Russet Burbank is a variety genetically predisposed to tuber impact damage and subsequent bruising compared with a more mechanically resistant variety such as Cara (Croy *et al*. 1998, Johnson *et al.* 2003). This is reflected in an enhanced oxidative burst in the susceptible variety following impact and is the basis of a sustainable correlation between level of bruising and rate of radical generation (Johnson *et al.,* 2003). It is of interest therefore to note that the levels of elicitor derived from the separated pectic fractions are consistently higher from the cell walls of the mechanically susceptible variety. Whether this is the result of qualitative differences in the nature of the pectic oligosaccharides or a quantitative effect remains to be elucidated but this is the first report of a possible correlation between a structural component and a mechanical property in potato tuber cells.

Overall there was a significant discrepancy between the elicitor levels generated by superoxide from the starting total cell wall material and the total recovered in the separated pectic fractions. This is most likely a consequence of differences in physical or chemical organisation of the pectins in the cell wall which limits superoxide scission. Extraction of the cell walls with the EPG/PME enzymes releases pectin poly- and oligo-saccharides (fraction 1) which can act as an elicitor without radical treatment. It is reasonable to assume that some of these elicitor molecules would not be created by the action of superoxide on the same pectic polysaccharides but may be modified by the action of the radicals. This would account for the observed diminishment in elicitor content of pectic fraction 1 on treatment with superoxide which destroys elicitors or diminishes their efficacy in inducing an oxidative burst. In contrast the elicitor content of pectic fraction 2 was significantly enhanced by superoxide treatment resulting in a 2-5 fold increase in elicitor activity. According to Sorensen *et al*. (2000) this fraction accounts for only about 16% of the total extractable pectin yet is the source of most of the radical-generated elicitor. Clearly there are interesting differences between the structures of the pectins in the separated fractions which may spawn a heterogeneous mixture of elicitor sizes and types as the result of radical scission.

Further evidence for heterogeneity comes from the size fractionation of the radical-generated elicitors by molecular filtration (figure 9). These results indicate that elicitor activity was split between the two sized fractions with 80% of the activity was <5kDa even after extensive washing almost 20% of the elicitor activity was retained with a mass > 5kDa. Again this indicates that some pectin elements despite being solubilised by superoxide action are resistant to further radical scission.

The elicitor content of different tissues released by radical scission, varies enormously indicating clear compositional differences. There are significant differences between stolon end and rose end with the latter showing about 50% less. There are also differences between the outer (cortex) tissues and the inner (medulla) tissues with very low levels released in the latter tissues. No elicitors were released from purified starch grains isolated from stolon cortex tissue presumably reflecting a clean preparation free from cell walls. Such compositional differences may reflect differences in the mechanical properties of the cells in these tissues as has been observed in other plant materials.

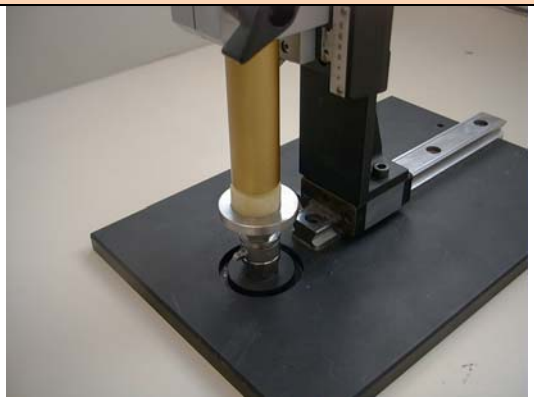
Conclusions: Plant cells respond to invading pathogens with a localised oxidative burst (Brown *et al.* 1998). The biphasic response in plant-pathogen systems comprises an initial non-specific stress response to the pathogen challenge followed by a specific pathogen-plant interaction leading to a second generation of active oxygen species. It is interesting that the mechanical impact induced system described here also stimulates a localised apoplastic biphasic oxidative burst. We therefore propose a model in which potato tuber cells respond to an abiotic mechanical stimulus by rapid flux of calcium and potassium ions. This in turn leads to protein kinase C cascade activation leading to generation of superoxide radicals via an NADPH-oxidase like enzymatic system. This signalling is progressive and builds up to a maximal level around 2 hours post stimulus. Whilst the majority of superoxide (and interconverted radicals) are rapidly detoxified some are able to cause oxidative scission of polysaccharide components of the plant cell wall. Scission generates a range of bioactive oligosaccharide elicitors and whilst the superoxide being generated from the initial response begins to wane more of the elicitors are beginning to bind to receptors in the cells. This elicitor-receptor binding triggers the ion flux involving potassium and calcium again followed by protein kinase cascade activation leading to a second sustained release of superoxide. The second phase is initiated within a few minutes of elicitor binding and has a duration of around 100 minutes. According to our model any such apoplastic generation of radicals should lead to scission of pectic polysaccharides in susceptible cell walls leading to a further oxidative burst and could provide an alternative explanation for the biphasic oxidative burst seen in plant-pathogen interactions.

5.3 - Tuber tissue physical properties - energy transmission

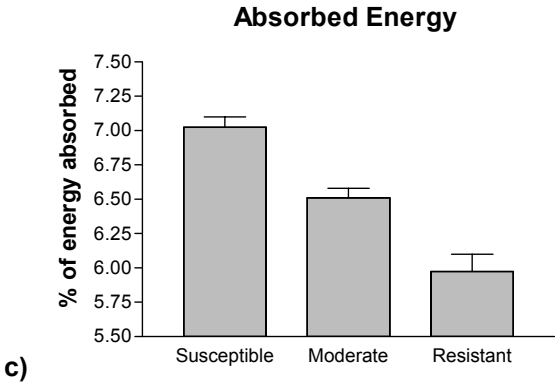
This experiment has analysed a range of tuber slices to ascertain the levels of energy transmission through a defined tuber thickness. Measurements indicate that bruise susceptible varieties tend to transmit less of the energy of impact (~5%) compared to the resistant varieties which transmit more of the energy (~8%). Varieties of intermediate susceptibility give values between the two extremes. These experiments have tentatively suggested that bruise susceptible tissues absorb more energy than their bruise resistant counterparts, strongly indicating a physical component (eg cell wall composition or cell turgor) linked to bruise susceptibility.

By inserting a slice of potato tuber between the impact head and the load sensor it is possible to ascertain how much energy is transmitted through the tuber tissue. The results, albeit preliminary, suggest that there is a difference between bruise susceptible and bruise resistant varieties. Energy absorbed by 0.5cm tissue from tubers displaying different mechanical properties

Figure 5.12 Tuber tissue physical properties - energy transmission/absorption

a) 

b) 

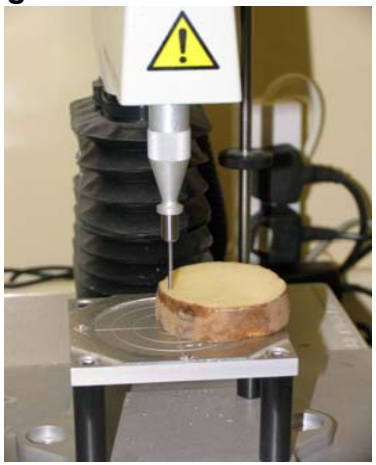
c) 

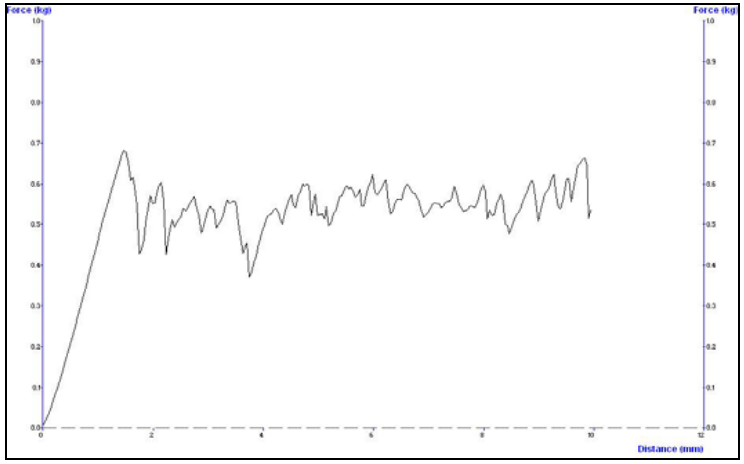
Conclusions: Bruise susceptible varieties tend to absorb more energy than bruise resistant varieties which transmit the energy through the tissue more efficiently. This would make sense if the energy of impact was absorbed by components in the susceptible tuber tissues causing internal disruption. Resistant varieties, perhaps with a more mechanically rigid cell wall network may be able to transmit the energy from the impact throughout the tuber without much being absorbed.

Recommendations: As a simple comparative method this produced some interesting data albeit with only three varieties. The potential for a simple physical method for assessing bruise/mechanical susceptibility is worthy of further investigation to establish if susceptible and resistant varieties show consistent levels of energy absorbed/transmitted. Further studies would need to include tubers of different hydration status (turgor) which might be expected to influence the amount of absorbed energy.

5.4 - Tuber tissue physical properties – penetrating force

a) 

b) 

c) 

d)

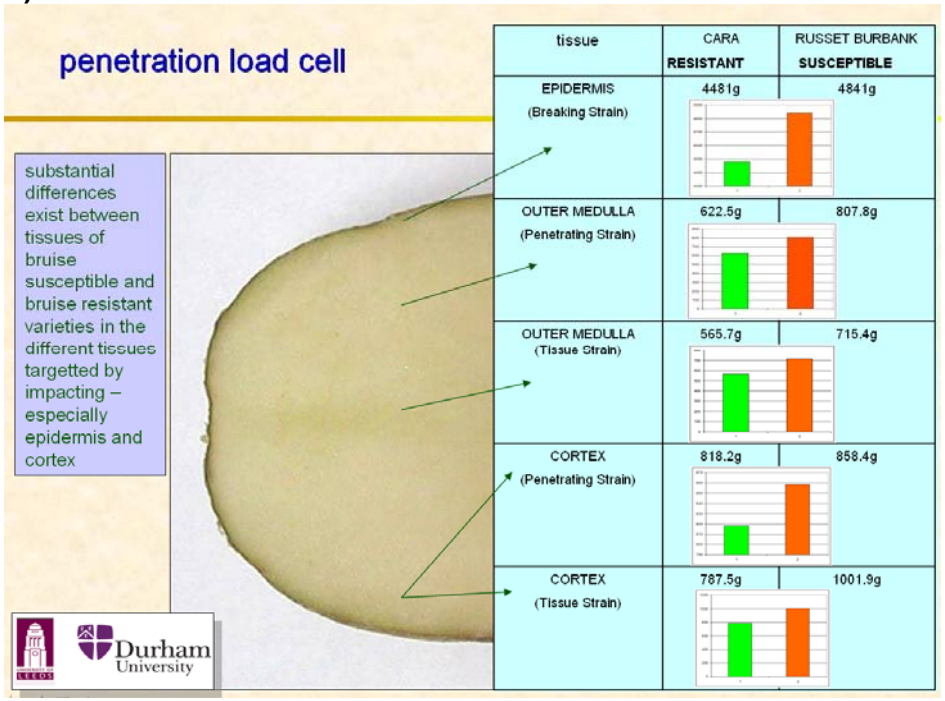


Figure 5.13 - Tuber tissue physical properties – penetrating force

a) This work was carried out in collaboration with the Department of Food Technology at the University of Leeds with thanks to Dr Jianshe Chen and colleagues. They use highly sensitive physical techniques for measuring the 'crispness' of processed foods such as crisps and biscuits. **b)** One of these techniques involves advancing a metal probe attached to a load cell, at a precise, constant speed so that it penetrates the test tissue – in this case selected tuber samples. **c)** The force (Kg) required to maintain this penetration is measured and plotted against the distance the probe has penetrated (mm) into the tuber tissue. **d)** Different tissues can be investigated in the same sample and many experiments can be carried out in a relatively short time. The preliminary results shown opposite **(d)** were very promising – in comparisons between tissues of *Cara* (resistant) and *Russet Burbank* (susceptible) the penetration load measurements were significantly higher for the susceptible tuber tissues requiring up to 30% more force to maintain the same penetration speed. Exactly what this means in terms of tissue and cell structure will require further investigation but basically the results indicate that susceptible tissue is 'crisper' and less pliable than the resistant variety, perhaps because of differences in cell wall composition (pectic content). Equally it could also indicate that the cells are more turgid in susceptible tubers. When exposed to an impact a susceptible variety is unable to disperse the force and the cells are ruptured causing bruising. The resistant tuber can more easily disperse the energy of the impact through the cell walls or the cells themselves without causing disruption.

Conclusions: In essence it has been demonstrated that with increasing susceptibility to bruising there is an increased mechanical resistance observed within the tuber tissue. This was measured using a probing load sensor on epidermal, cortical and medullary tissue. All three tissue types demonstrated the increasing mechanical resistance with increasing susceptibility. More work will be carried out to confirm these results along with experiments designed to test how strong the correlation is.

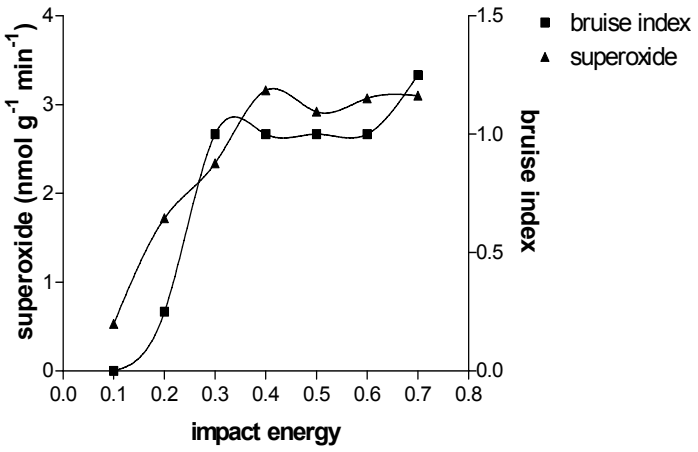
Recommendation: As a simple comparative method this produced some interesting data albeit with only three test varieties. The potential for a simple physical method for assessing bruise/mechanical susceptibility is worthy of further investigation to establish if susceptible and resistant varieties show consistent levels of energy absorbed/transmitted. Further studies would need to include studies on tubers of different varieties and of different hydration status (turgor) which might be expected to influence the amount of absorbed energy measured by this technique. Should valid correlations be confirmed further work would be required to see if a portable, more economic version of the instrumentation could be developed.

Although only preliminary studies have been undertaken (necessarily due to a lack of time), these physical methods which investigate the structural properties of tuber tissues are promising approaches to reveal correlations between the physical attributes of tubers and mechanical damage and bruising. In conjunction with the biochemical studies on purified cell walls (**Section 5.2 – Dissection of the biochemical responses**) and the completion of the transcriptomic analyses (**Section 6 - Molecular Genetic Studies – transcript profiling**) a better understanding of the factors influencing bruise susceptibility should be possible.

5.5 – Further studies on the biochemistry of the impact response

Superoxide generation at low impact energies - The two preceding, preliminary studies have suggested structural differences in tuber tissue materials that could be the origin of the differences in the physical properties of the tuber tissues leading to variation in bruise susceptibility. In a series of experiments designed to see if there is a threshold force below which there is no detectable increased SO radical generation and if so do the responses of SO generation and bruise pigment synthesis correspond. It is already known that the bruise response is affected by the degree of impact force and there is a threshold below which no bruising takes place. As shown in **Figure 5.14**, by progressively diminishing the energy of impact on a series of *Russet Burbank* tubers it was possible to demonstrate that a threshold level is reached below which superoxide synthesis takes place but bruise pigment formation does not. This suggests that even when the impact is insufficient to cause membrane disruption (leading to bruise pigment synthesis), there is a sufficient perturbation to the cellular systems to still induce a superoxide burst (enhanced SO generation). The graph below clearly shows that at an impact energy of 0.2J or less there is almost no pigment formation (as measured by bruise index) but an increased level of superoxide generation. Thus at low energy of impact levels of mechanical damage leading to bruising are independent of SO generation. As levels increase >0.2J the two phenomena become synchronized and it appears that they are interdependent – hence the suggestion that superoxide radicals rather than molecular oxygen may be the preferred molecular form of oxygen utilized by the enzyme polyphenol oxidase which is responsible for the early oxidative reactions leading to melanin (bruise) pigment synthesis. An interesting question arises from these experiments as to why for a given tissue core, the generation of SO increases in an exponential fashion. The answer appears to be that at low energy not all of the cells within the sampled core have been activated or have been impacted above the threshold. The SO generation then reaches a plateau – ie no further increase in SO generation with further increases in energy of impact. Here it is likely that all the cells that can be activated and detectable by the XTT assay have been activated. The abrupt onset of pigment generation could be interpreted as indicating that at this specific energy most of the cells collapse therefore all the cells in this tissue are of similar strength? Similar to the SO generation phenomenon as the energy of impact increases bruise pigment synthesis slows and also reaches a plateau. What is clear is that SO generation commences at lower energy of impact which might be expected since SO generation is an active process requiring the integrity and presumably continued viability of the cells. Higher energies might kill them off; pigment synthesis results from cells which have been disrupted by the impact and therefore have been killed off.

Figure 5.14 - Superoxide generation at low impact energies. At impact energies below about 0.2J bruise damage cannot be detected but SO generation is significantly enhanced.

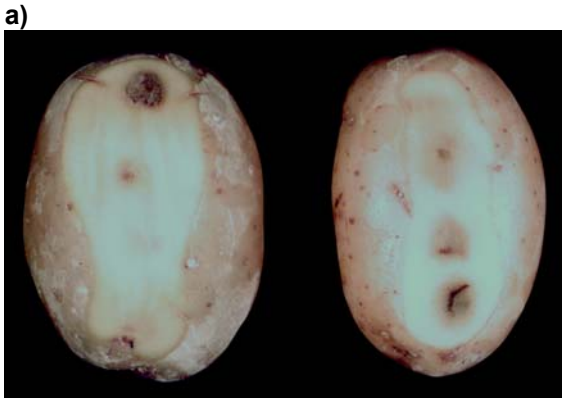


Conclusions: SO generation is initiated at a lower energy of impact than the energy required to damage tuber cells to the extent that they commence pigment synthesis. These results point to fundamental differences in the triggers instigating SO generation and bruise pigment synthesis.
Recommendations: Further investigation of the nature and distribution of wholly and partly damaged cells within the impact zone. This could have fundamental importance in understanding how the energy of impact is dispersed throughout the underlying peridermal tuber tissues. For example does the impact volume and 'shape' change in varieties displaying different bruise susceptibilities. The answers to these questions could influence the way in which tubers might be impacted to diagnose bruise susceptibility.

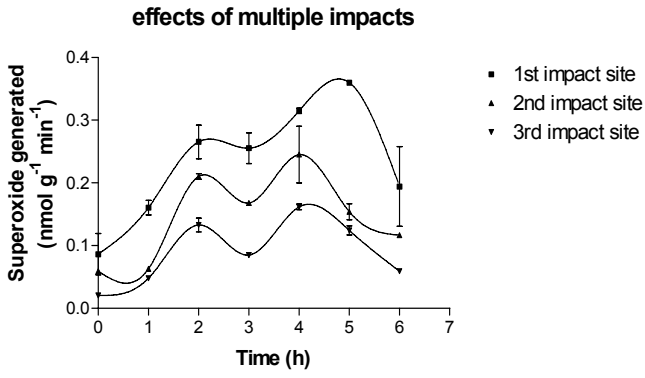
Bruising and superoxide radical generation following multiple impacts and membrane depolarization: An intriguing result was discovered during a series of test experiments to increase the quantity of impacted tissue for biochemical experiments. The objective was to maximize the number of impacted tissue cores that could be removed from single tubers thereby diminishing the quantity of potatoes required for these experiments. The preliminary studies using the standard bruise susceptible varieties showed that after an initial standard impact the normal intense bruise developed after 24-48h as expected. However when tubers were exposed to several standard impacts in rapid succession (i.e. seconds between each impact) while the first impact produced the expected bruise the subsequent impacts showed very much diminished bruise response (**Figure 5.15a**). The phenomenon indicates that the first impact 'immunises' the tuber and protects it from further damage responses. The question arose then as to whether the same multiple impact phenomenon affected the tuber tissue at the level of SO generation. **Figure 5.15b**) shows that it does influence SO generation right across the biphasic response. Furthermore the effect when quantified is found to be cumulative – that is each further impact becomes less effective in causing increased SO generation. A further series of experiments with a range of different varieties showed that the phenomenon applied more or less to the whole tuber – impacting at one end of the tuber affected the subsequent bruise responses at the other end irrespective of which end was impacted first (**Table 5.2**). These results provide new information on the molecular events taking place following impact damage.

Figure 5.15 - Bruising and superoxide radical generation following multiple impacts.

a)



b)



about the way in which forces are transmitted through tuber tissues but more importantly provides a new insight into the primary and immediate effect of the impact on the affected cells. From the literature the response of cells to physical pressure has implicated ion channel proteins and so-called 'stretch receptors'. It is unclear how these function but ion-channel proteins involved in the transport of charged species across cell membranes appear to function as molecular pumps changing their configuration depending on whether they are loaded with an ion or unloaded. A follow on experiment was set up to see if exposing tuber cells to a preliminary electric field (depolarisation) would affect their subsequent impact responses. Although this was a purely arbitrary experiment it did in fact show that the SO radical generation was significantly reduced by up to 12% and opens up several possibilities for future investigations into how the cells are responding to impact.

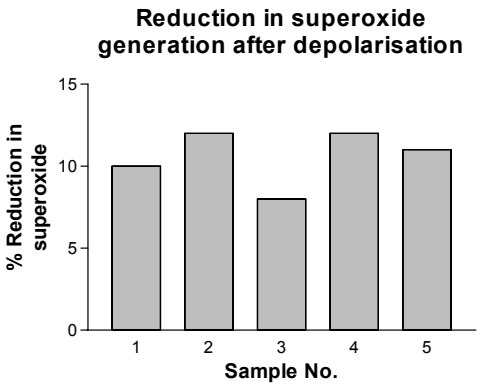
Table 5.2 Bruise index values for bruises arising from multiple impacts

Variety	1st Stolon	2nd Bud	1st Bud	2nd Stolon
Russet Burbank	10.4 ± 0.9	6.3 ± 1.1	8.5 ± 0.9	3.9 ± 1.1
Saturna	9.8 ± 1.2	5.7 ± 0.6	7.7 ± 1.1	2.9 ± 1.3
Cara	6.3 ± 0.8	3.5 ± 0.9	6.0 ± 0.4	2.6 ± 0.9
King Edward	5.0 ± 0.8	2.8 ± 1.0	3.5 ± 1.4	1.4 ± 0.8
Maris Piper	2.9 ± 1.0	1.2 ± 0.5	1.8 ± 0.3	0.9 ± 0.3

Figure 5.16 – Influence of depolarization on superoxide radical generation.

a)

b)



Conclusions: Tubers comprise of a mass of cells connected by a network of cell walls. Physical forces applied at any part of the tuber will transmit the force through this network to all parts of the tuber. Cells will be affected in the close vicinity to the point of impact but those cells in the remote parts of the tuber will also be affected through the transmitted energy. This is reflected in the bruise response as well as the radical generation throughout the biphasic response. Most interestingly the results indicate that once triggered initially, subsequent responses are diminished and could indicate a phenomenon of membrane depolarization. Indeed preliminary experiments in which tubers were exposed to electric fields showed that SO generation was significantly reduced.

Recommendation: As with the other biochemical and physiological experiments these preliminary investigations are intriguing and yielded totally novel results. Further investigations could shed light on the molecular mechanism which sets off bruising and also the signalling leading to SO radical generation.

Part 6 - Molecular Genetic Studies – transcript profiling

Objective 6 – Molecular genetics
Task 20 – Potato samples selected and extracted
Task 21 – Microarray preparation and assays

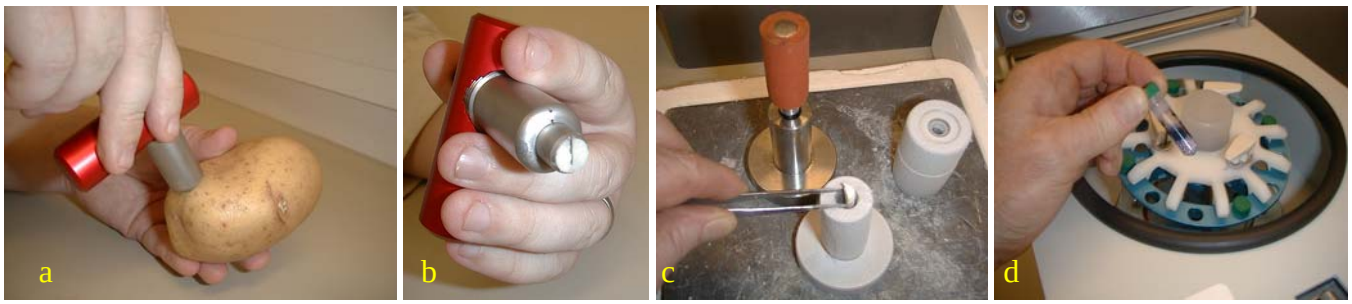
The experimental work on this part of the LINK project was primarily carried out during 2006-2007 by Dr Ron Croy (Durham) and Dr Francesca Fontana (University of Pisa) in collaboration with TIGR (The Institute for Genomic Research) within the NSF Potato Genome Project Consortium in the US. Potato tuber materials for the work were collected primarily during the 2003 and 2004 field trials as described below.

6.1 Introduction: Bruise susceptibility is the consequence of complex interactions between genetics and the environment. This can be seen in different potato varieties display a greater or lesser predisposition towards susceptibility or resistance to mechanical damage and bruising. Bruising is the result of mechanical damage in a susceptible variety which possesses the appropriate biochemical components
The same varieties behave differently from one growing season to the next indicating that environmental conditions modulate the expression of the genetics of the variety. All of this forms the philosophy behind a detailed investigation of gene expression in tubers of different varieties displaying different bruising predispositions. Transcript profiling uses a large collection of characterized gene probes to compare the expression of potato genes in different tuber samples. The probes are robotically arrayed as minute (50-100µ diam) spots of DNA onto glass slides known as microarrays. Potato microarrays typically contained more than 10,000 gene sequences and were prepared by TIGR (The Institute for Genomic Research) consortium potato genomics in the US. The tuber harvesting was carried out within the project period, the RNA isolations and the actual transcript profiling were carried out during 2006 after the project period had officially finished. The microarray hybridizations and data acquisition were carried out in late 2006. (Contact Rensink)

6.2 Methods - Research plan and methodology

Potato varieties were selected on the basis of their expected genetic predisposition towards a particular bruise status on the scale of very susceptible (eg *Russet Burbank*) through to resistant (eg *Cara*) (Table 6.1). The varieties were grown during the 2003 and 2004 seasons in quadruplicate, randomised 24m^2 field plots at ADAS Gleadthorpe, Nottinghamshire, UK, under carefully controlled agronomic conditions. Since all varieties were grown in the same location and at the same time of year differential effects from the growing conditions should have been minimised within each growing season. Some plots had restricted potassium nutrition which promotes bruise susceptibility while the remainder had applications of standard fertilizer. Agronomic and growth parameters were monitored throughout the developmental period from planting through to defoliation, harvest and once in storage. The actual bruise status of the harvested tubers was determined post-harvest by standard impact tests and by superoxide assay (Johnson *et al* 2003) (Table 6.1). Throughout the two growing seasons the expected bruise status of the selected varieties was observed.
Tuber samples, representative of each selected field plot, were manually harvested, washed thoroughly several times in water, then in sterile detergent solution (2% SDS) in pure water and finally rinsed in sterile water. Tissue samples taken from the stolon end of the tubers and immediately frozen in liquid N_2 (harvesting to frozen < 1h). The stolon cortex is the most bruise susceptible tissue in the tuber and in this study comparisons between varieties employed samples taken from this region only to minimize tissue variation.

Figure 6.1 - Tuber RNA extraction. a) Excising sample tissues from the stolon end of tuber samples using a coring device. b) This rapidly excises two half cores of cortex tissue which are ejected directly into liquid nitrogen for RNA preparations. c) Frozen cores were pulverised in a stainless steel mortar and pestle and d) tuber total RNA was extracted using a semi-automated system (FastPrep FP120) or by conventional methods.



Standard cores were excised using the Mk 3 coring device (**Figure 6.1a,b**). Larger tuber tissue samples were cut from the stolon ends of the sampled tubers with a sterile knife, sub-divided into smaller pieces and frozen. All samples were transported and stored in liquid N_2 prior to RNA extraction. Frozen tissue cores or were pulverised to a frozen powder in a stainless steel mortar and pestle device (**Figure 6.1c**) and RNA's extracted from all tuber samples according to the TIGR protocols using hot acidic phenol (SGED_SOP_3.2.1) or FastRNAPro™ (Qbiogene). For larger samples up to 4g of tuber tissue were pulverized in liquid nitrogen in a mortar and pestle before extraction using the hot acidic phenol method. Both of these methods produced good quality, undegraded RNA (**figure 6.2**). The quality and quantity of RNA were assessed by UV absorbance spectra, $E_{260/280}$ ratio (**table 6.1**) in a Beckman DU7500 diode array spectrophotometer and by formaldehyde gel electrophoresis (**figure 6.2**). RNA preparations were stored in liquid nitrogen until required; the RNA samples in dry ice were transported to TIGR in the US by courier. RNA preparations which did not meet the required purity were re-purified or replaced with a new preparation. The quantities of tuber tissue collected and stored in liquid nitrogen was adequate for the large amounts of RNA required by TIGR for the hybridisations (at least 120µg total RNA / hybridisation). The materials used for these analyses included samples from different plants, field plots or growing seasons so were representative biological replicates of the same experiments. Microarray hybridisations were set up to compare combinations of transcript populations from tuber samples selected from the available material listed in **table 6.1**. Since this investigation looked at a property inherent in the genetics of each variety rather than an 'experimental treatment', the 'control' and 'query' were varied depending on the comparison required. Within the TIGR-limited numbers of hybridisations the comparisons performed are shown in **table 6.2** along with the rationale for selecting these ones out of the many possible permutations. The comparisons between Cara (R) and Cultra (S) were important since these cultivars displayed significantly different bruise statuses but are closely related genetically (Cara is a parent of Cultra) which should help minimise non-bruise associated, varietal variation. Comparisons of the patterns of gene expression between selected pairs of samples should reveal gene sets common to susceptible and to resistant varieties and those that differ significantly between varieties. For example, assays 1+2, 3+4, 17+18, 19+20, 25+26 and 27+28 will reveal patterns of gene activity significantly different between susceptible and resistant tubers; 13+14 and 21+22 will compare abundant expression between different susceptible varieties; 29+30 will compare abundant expression in different resistant varieties; the other hybridisations represent comparisons with moderately resistant materials to reveal quantitative differences in the genes expressed differentially between very susceptible or resistant varieties.

Table 6.1 – tuber samples harvested			
Sample	**Bruise class***	**Year**	**Bruise Status (new test)**
Russet Burbank	VS	2004	7.63
Russet Burbank	VS	"	7.87
Cara	R	"	2.91
Pentland Crown	R2	"	4.21
Cultra	S	"	6.13
King Edward	R3	"	3.94
Cultra	S	"	6.10
Cara	R	"	2.62
Cultra	S	2003	6.66
Cara	R	"	3.19
King Edward	M2	"	5.22
Cara	R	"	3.49
Pentland Crown	M1	"	5.06
Cara	R	"	3.28
Cultra	S	"	6.27
Cara	R	"	3.83
King Edward	M2	"	5.61
Cultra	S	"	6.22
Cultra	S	"	7.01
Pentland Crown	M1	"	4.85

Bruise Status Colour Key:

VS = VERY SUSCEPTIBLE	7.6 - 10
S = SUSCEPTIBLE	5.6 – 7.6
M = MODERATE	4.6 – 5.4
R = RESISTANT	2.6 – 4.5

Since insufficient RNA was isolated from PC2003 and KE2003 to use only one of these for all the hybridisations of a **moderate** susceptibility cultivar (only enough RNA was available for 7 hybridisations with each. Thus both RNA's needed to be used. So it was decided that the best way to avoid complications with varietal differences was to use both in comparable hybridisations and not to use any duplicates. The tables opposite and below reflect the modified hybridisations incorporating M1 (PC2003) and M2 (KE2003). Note that between 2003 and 2004 Pentland Crown and King Edward increased from moderate to resistant. Comparisons were set up to examine this.

* **Bruise class** is the average predisposition to bruise damage assigned to individual potato varieties (The European Cultivated Potato Database).

** **Bruise status** (new test) is the classification based on the superoxide generation test.

TIGR Microarrays and trancript analysis

The TIGR potato "10K version 4" cDNA microarrays were used for these experiments. The cDNA's used as expression probes on the microarrays were derived from the potato stolon, root, microtuber, dormant tuber, germinating eye, healthy leaf, and Phytophthora infestans-challenged libraries (incompatible and compatible) sequenced at TIGR in collaboration with others on the NSF Potato Functional Genomics Project. A total of 15,264 cDNAs were selected for the microarray including a set of clones selected for controls on the arrays. Positive and negative controls consisted of 16 constitutive potato gene probes, tomato gene probes, *Phytophthora infestans* sequences (potato blight pathogen), and other bacterial genes / plasmids plus 10 human cDNA's/EST's. Microarrays were printed on Corning Ultra Gaps Slides using an *Intelligent Automation Systems* arrayer equipped with *Telechem Stealth Microspotting Pins* arranged in a 12 x 4 pattern and providing matrices of 26 x 26 = 676 probe spots. All 15,264 cDNAs were spotted in duplicate on the slides making a total of 32,448 total spots. Two of these microarrays were used independently for each of hybridization set (target pairs) described in **table 6.2** which along with reciprocal target hybridisations and the duplicated probes (DNA spots), essentially provided octuplicate analyses of the gene probes. Labeled targets were prepared from each of the purified tuber RNA samples (**table 6.1**) as fluorescent cDNAs prepared by reverse transcription, incorporating a cyanine dye, either Cy3 (green fluorescence) or Cy5 (red fluorescence). In this way the relative binding of either target to each probe can be distinguished and individually quantified to provide a measure of the differential gene expression in the two targets. For each of the hybridization pairs (table 6.2) aliquots of the relevant fluorescent targets were mixed and hybridized to one microarray under standardized conditions. Following hybridization and washing all the slides were scanned with a GenePix Array Laser Scanner at 532nm and 635 nm for the two fluorescent dyes (Cy-3 and Cy-5) and separate gray scale image files were generated for each channel (Cy3 and Cy5). Finally a composite overlay image of both channels was generated and in the resulting pseudocoloured image in which the green Cy3 and red Cy5 signals are overlaid -- yellow spots indicate equal intensity for the dyes. Image analysis software is used to determine signal intensities for each dye bound to each probe on the array and the ratio of Cy3 intensity to Cy5 intensity (Cy3/Cy5) is calculated. High ratios indicate relative excess of the transcript (i.e. a higher level of probe gene expression) in the Cy3-labeled sample, and low ratios indicate relative excess of the transcript in the Cy5-labeled sample. Values near one indicate equal abundance, equal levels of gene expression in both samples. To facilitate visualization of the genes differentially expressed (ie at the two ratio extremes Log_2(Cy3/Cy5) is calculated and (See example set Once all the microarrays have been analysed the relative levels of expression of the different genes in each set of hybridizations can be compared and correlated with the bruise status of the original tuber samples. Cluster analysis of these data will generate the gene expression patterns correlating with bruise susceptibility or resistance. Candidate diagnostic gene sets identified in this study will require to be tested in further field trials by additional microarray analyses and ultimately by real time-PCR before firm conclusions can be drawn.

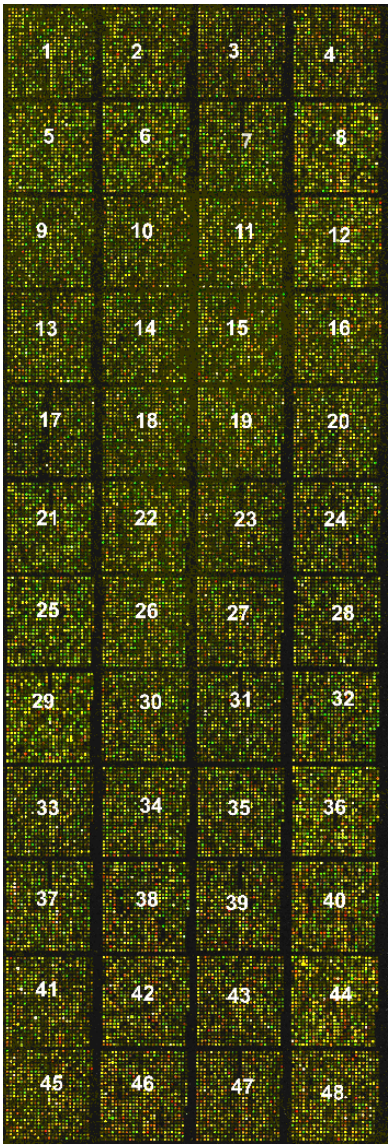


Figure 6.2 - TIGR 10K, version 4 cDNA microarrays 48 blocks of 676 probe spots printed in the TIGR potato microarrays containing 15,264 potato cDNA's and control probes.

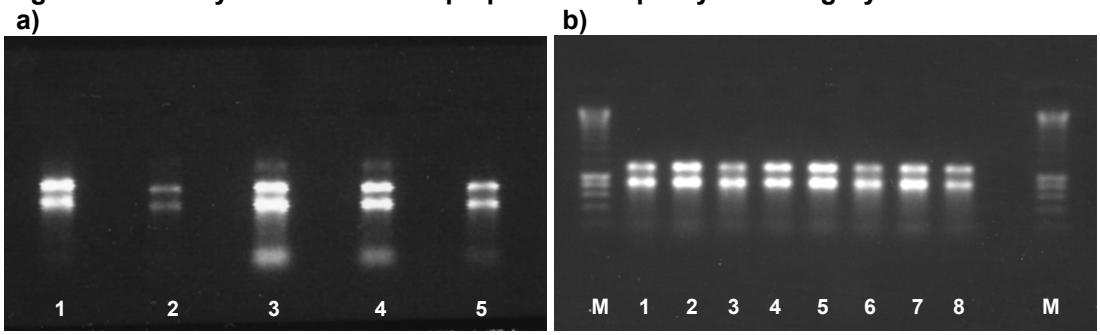
6.3 Results

Tuber RNA yield and quality: Tuber tissue was processed using the TIGR acidic phenol extraction procedure as outlined in the Methods (**Part 6.2**). The amount of RNA isolated (E_{254nm}) and its purity ($E_{260nm/}$ E_{280nm} absorbance ratio) were estimated by scanning the samples in a UV spectrophotometer as described in the Methods. The results obtained are presented in **Table 6.2**

Table 6.2 – Analysis of tuber RNA preparations: yield and purity

variety	overall yield	$E_{260nm/}$ E_{280nm}	concentration
Cara	1040 µg	2.0	2.6µg/µl
Cultra	780 µg	2.0	2.6µg/µl
Russet Burbank	1000 µg	2.0	2.0µg /µl
Pentland Crown	414 µg	2.0	2.0µg /µl
King Edward	492 µg	2.0	2.5µg /µl

Figure 6.3: Analysis of tuber RNA preparations – purity and integrity



Results of a number of trial tuber RNA extraction methods tested on the same tuber materials (See description in the Methods section). The criteria used to judge the efficacy of the method were based on 1) UV absorbance (E_{254nm}) to quantify the yield; 2) the $E_{260nm/}$ E_{280nm} absorbance ratio which indicates purity and 3) the qualitative appearance on electrophoretic gels as shown here. Sample 1 in track 1 (prepared using the TIGR – phenol extraction method) was ideal giving a good yield, high $E_{260nm/}$ E_{280nm} absorbance ratio (1.9 > 2.0) and good intact rRNA bands of equal intensity (arrowed) and no evidence of the RNA degradation as seen in samples 3 and 4 which also contain contaminating DNA.

The optimised method was used to extract RNA from a range of different tuber samples (different varieties, different growing seasons) which had been stored at -196˚C (liquid nitrogen) for periods of up to 3 years. The final experimental details of the optimised method are provided in appendix III. This method was chosen for all subsequent RNA isolations within this project. Varieties extracted and analysed above were: 1) Pentland Crown 2) King Edward 3) Pentland Crown 4) King Edward 5) Cultra 6) Russett Burbank 7) Cara 8) Russett Burbank. Tubers were sampled just prior to crop harvesting (24h) so were at or nearly mature.

Microarray analysis

TIGR sample quality control and assessment: The tuber RNA samples arrived intact and on time at TIGR. Upon receipt the RNA samples were quality controlled by RNA electrophoresis and by UV absorbance and were judged to be suitable quality and quantity for the proposed microarray analysis. The samples were stored in liquid nitrogen until required. There was a significant delay between receipt of the RNA samples from Durham and the commencement of the microarray assays at TIGR. This was due to staffing and training problems at TIGR. The final microarray raw data and results were only received towards the end of 2006. Processing of the data has been limited due to software and time limitations.

Selection and rationale for microarray hybridizations: TIGR placed an upper limit on each collaborative project of 30 hybridizations

Table 6.3 – Microarray hybridizations and rationale

The hybridisations devised for microarray analysis by TIGR. All hybridisations were performed in duplicate and include some reciprocal labelling (Cy3/Cy5 and Cy5/Cy3) combinations; all of the probe spots are duplicated in the microarrays; so this effectively provides octuplicate assays for each gene (it is generally recommended to perform quadruplicate microarray analyses). For each hybridisation probes from two RNA preparations were used simultaneously. To distinguish between the two one was labeled with Cy3 and the other with Cy5 as outlined in the **Part 6.2**. The column on the right describes the differential gene expression investigated by each hybridisation. ** Preliminary analyses of the results from these comparisons are shown below.

hyb	control	query	investigates differential expression between:
1	R	VS	resistant vs very susceptible *cv* **
2	R	VS	duplicate of 1 **
3	R	S	resistant vs susceptible (*cf* 1+2) *cv*
4	R	S	duplicate of 3
5	R	M1	resistant vs moderate *cv*1 (*cf* 1+2+6)
6	R	M2	resistant vs moderate *cv*2 (*cf* 1+2+5)
7	M1	VS	moderate *cv*1 vs very susceptible *cv* (*cf* 1+2+8)
8	M2	VS	moderate *cv*2 vs very susceptible *cv* (*cf* 1+2+7)
9	M1	S	moderate *cv*1 vs susceptible *cv* (*cf* 3+4+10)
10	M2	S	moderate *cv*2 vs susceptible *cv* (*cf* 3+4+9)
11	M1	R	moderate *cv*1 vs resistant *cv* (reciprocal of 5)
12	M2	R	moderate *cv*2 vs resistant *cv* (reciprocal of 6)
13	S	VS	susceptible vs very susceptible
14	S	VS	duplicate of 13
15	S	M1	susceptible vs moderate *cv*1 (reciprocal of 9)
16	S	M2	susceptible vs moderate *cv*2 (reciprocal of 10)
17	S	R	susceptible vs resistant *cv* (reciprocal of 3 & 4)
18	S	R	duplicate of 17
19	VS	R	very susceptible vs resistant *cv* (reciprocal of 1 & 2)
20	VS	R	duplicate of 19
21	VS	S	very susceptible vs susceptible *cv* (reciprocal of 13 & 14)
22	VS	S	duplicate of 21
23	VS	M1	very susceptible vs moderate *cv*1 (reciprocal of 7)
24	VS	M2	very susceptible vs moderate *cv*2 (reciprocal of 8)
25	VS	R4	very susceptible vs resistant but same *cv* (R4)
26	VS	R4	duplicate of 25
27	R4	VS	different resistant vs very susceptible *cv* (R4)
28	R4	VS	duplicate of 27
29	R	R2	resistant vs different resistant R2 *cv* (*cf* 31+32)
30	R2	R	different resistant R2 vs resistant *cv* (*cf* 3+4+10)
31	R	R3	resistant vs different resistant R3 *cv* (*cf* 31+32)
32	R3	R	different resistant R3 vs resistant *cv* (*cf* 3+4+10)

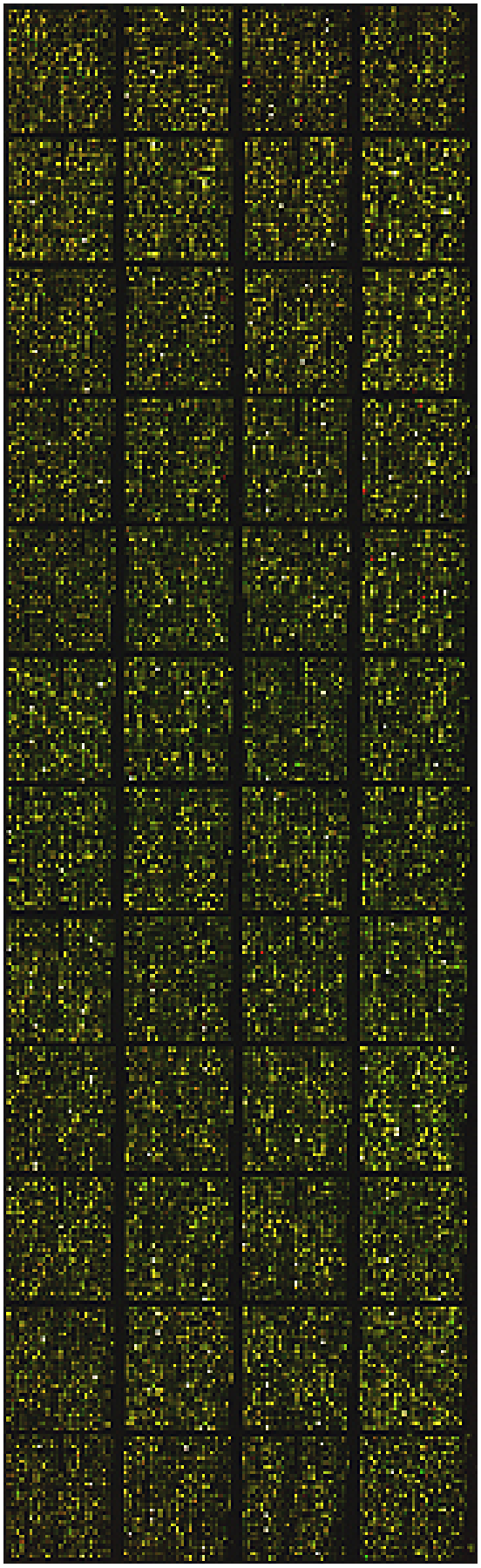
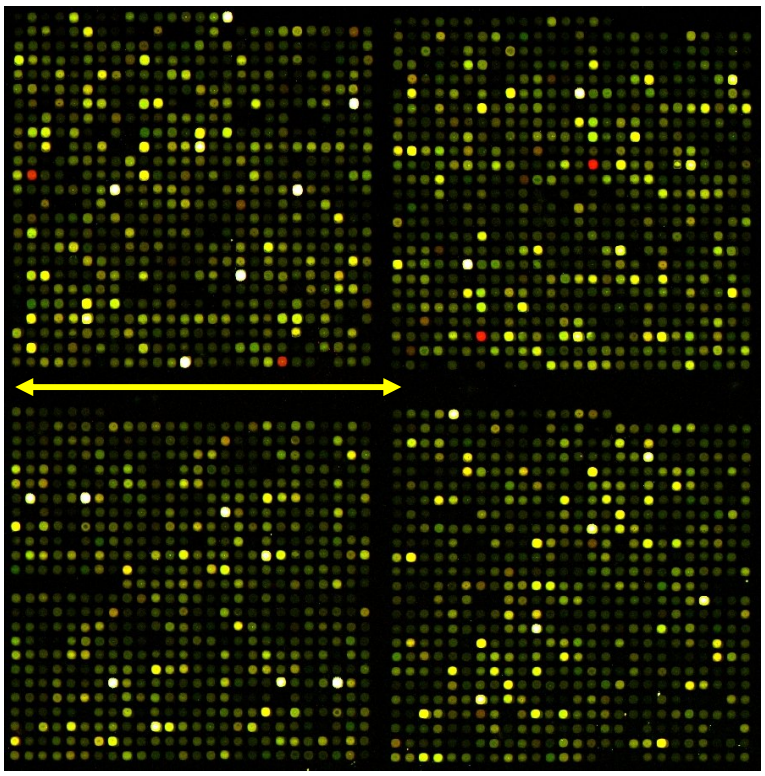


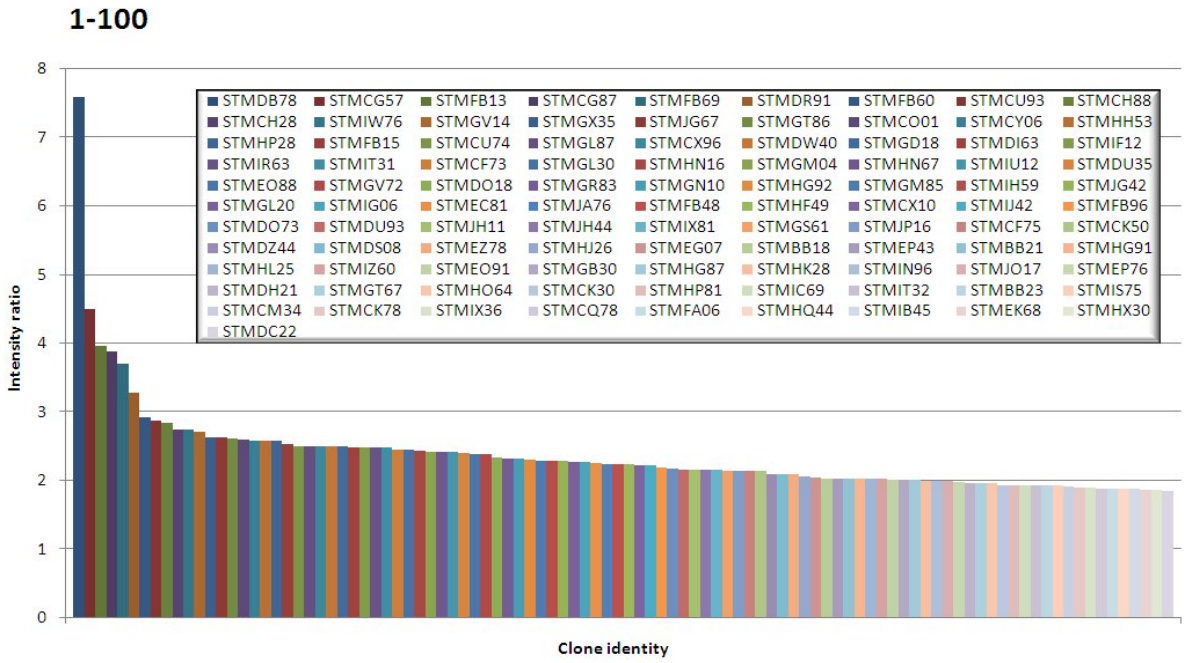
Figure 6.4 - Example microarray chip fluorescent scans
a) Left - An example superimposed and pseudocoloured images of a microarray analysis from this project showing a single TIGR potato gene chip hybridized with two tuber sample targets to elucidate differential gene expression in one of the comparisons listed in **Table 6.2.** Each chip comprises of more than 15,000 different gene probes printed as duplicate spots of DNA ~75-100 microns in diameter. Only one example of the thirty two images from the complete project is shown since at this level one analysis appears much the same as any other.



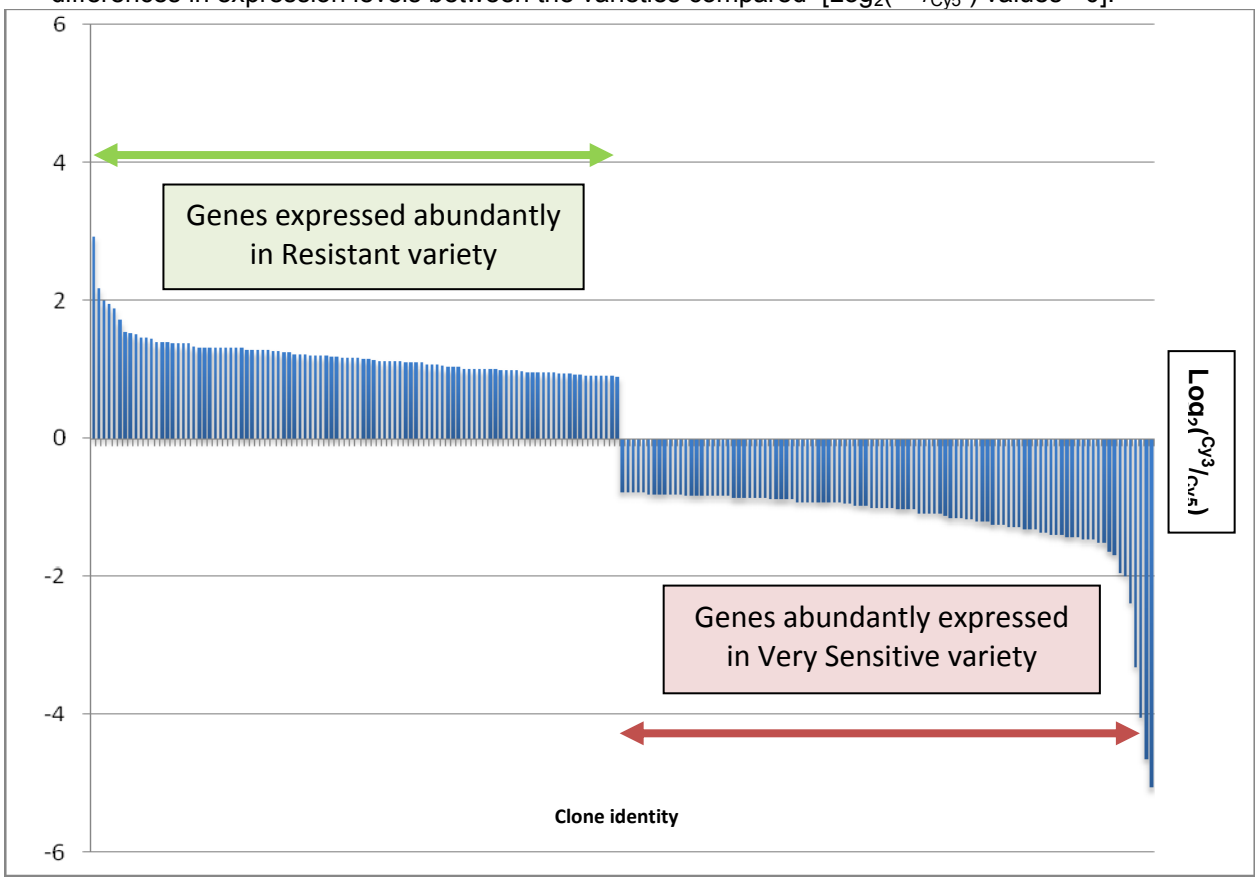
b) Above – An enlargement of a section of the hybridized gene chip showing the green, yellow and red spots corresponding to relative levels of gene expression in the RNA samples from two sets of potato tubers being compared (referred to as control and query samples). To be able to distinguish between genes expressed in each pair of samples the control RNA is labeled with a green fluorescent dye (Cy3) while the query RNA is labeled with a red fluorescent dye (Cy5). Following hybridization the chip is scanned with a red and a green laser and the fluorescence intensity measured for each spot. Green spots represent genes which are expressed in the query sample, red spots are genes expressed in the reference sample while yellow spots represent genes expressed in both samples. The measurement of the intensities and the normalization processes (e.g. to take into account different efficiencies of dye labeling, hybridization efficiency) are all carefully controlled before the results recorded and relative gene expression levels calculated as fluorescence ratios. The scale of the yellow arrow (distance between equivalent spots in a block) is 4500µ (microns).

Figure 6.5 - Microarray analyses. Plots of the differential expression results of the genes expressed at a ratio of 2x or more between a bruise resistant and a very bruise susceptible variety (**Table 6.2**).

a) Plot of [$^{Cy3}/_{Cy5}$] (ratio of Cy3 to Cy5 fluorescence values) for each of first 100 clones (ordered)



b) Plot of $Log_2[^{Cy3}/_{Cy5}]$ for first and last 100 clones after ordering. Graph shows the spread of abundant gene expression at the two extremes resistant and very susceptible. Most genes show no significant differences in expression levels between the varieties compared [$Log_2(^{Cy3}/_{Cy5})$ values =0].



Conclusions: The tuber RNA samples prepared from the bruise susceptible, bruise moderate and bruise resistant samples covering the two growing seasons were of high quality and suitable for microarray analysis. This was independently assessed by TIGR scientists. The results obtained from the microarray analyses are of good quality showing clean and discrete hybridizations (**Figure 6.2**). There are some interesting initial observations made from the results spreadsheets however these observations are at best cursory and incomplete due to a lack of time and the necessary software for the data mining (e.g. *GeneSpring* or *ArrayStar*). A study of this nature within a much larger project is inevitably very preliminary and requires significantly more experimental and corroborative work before solid conclusions can be drawn. This would involve further microarray analyses followed by RT-PCR analyses on selected candidate genes using potato materials from dedicated field trials. The amount of work and time involved in this part of the project was underestimated and at the time of submission of the final report there was a significant amount of work on the microarray analyses and data mining still to be carried out and a considerable amount of data processing and bioinformatics to be done to assess the relevance of the findings. Preliminary analysis of the microarray results albeit by simple manual spreadsheet sorting and scrutiny provided some useful initial data and indicate that the microarray analyses appear to be reproducible (same candidate genes identified in independent assays) and sensible in that at least some of few genes identified so far might be expected to function in tuber tissue. These results however only represent less than 10% of the total data to be processed. However it would be erroneous to draw any conclusions from the small fraction of the results investigated so far or even to suggest any of the candidate genes indicated to have elevated or lowered levels of expression in one or two comparisons. In addition to completing the 32 analyses using appropriate data mining software to identify all the candidate genes and perform the necessary checks, these genes then need to be correlated with the bruise properties of the tuber samples used. A significant amount of bioinformatic study will then be required to put the genes 'into context' (i.e. what role(s) do the gene products play in tuber cells and is this likely to be relevant to any aspect of mechanical properties or bruise generation in tuber tissue). At this point discriminating between bruise predisposition and other non-bruise related varietal differences will need to be done. The final data will need to be collated and used to devise a model based on the roles played by the gene products (enzymes, enzyme inhibitors, transcription factors, structural proteins etc.).

Recommendations: It would be easy to underestimate the future practical potential of these studies because of their fundamental nature and perhaps to discount this approach because of its labour intensive nature and the expensive resources required. However this is one of only a few global and unbiased approaches (the others being proteomics and metabolomics) that can address the molecular basis of complex biological phenomena such as tuber development, mechanical properties and bruise susceptibility. Transcriptomic analysis has the greatest capacity to reveal the components responsible for the development of bruise susceptibility and the gene chips currently available to do this are even better than the TIGR 10K chips used for this study. Once elucidated gene expression patterns associated with bruise sensitivity or resistance could provide the basis for future diagnostic tests through quantification of specific genes, their enzyme products or the products of the enzymes. The transcriptomic analysis started here should be completed and the conclusions analysed in detail and published. This work should be extended to realize the full potential of the preliminary results and to elucidate the molecular bases of factors which ultimately affect tuber quality including a predisposition to bruise susceptibility and mechanical damage.

During 2006/7 a major LINK proposal *(FQI 17 Br – Strategies to determine markers for quality of processed potato food products)* which required several months preparation, was submitted to Defra, combining the expertise of 5 research institutes (ADAS, SCRI, SBEU, BioSS and Durham) and with the support of eight representative concerns from the potato industry. The proposal was to combine several 'omic' approaches to investigate potato development and tuber quality including bruise susceptibility. The transcriptomic analyses were to use the POCI 44k element 60-mer oligo microarray chip developed by various potato research groups and manufactured by Agilent and estimated to represent more than 75 % of the potato transcriptome (expressed potato genes). This oligochip promises to be the most robust chip ever developed for the study of potato gene function. Unfortunately although the proposal was given excellent reviews by the referees and judged high quality science, appropriate and timely it was not funded.

Part 7 - References

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Part 8 – Final Conclusions and Commentary:

Including some suggestions and comments by the Project Manager about the failure to establish a correlation between bruise susceptibility and superoxide generation in the field trial samples. These comments are not in any particular order or priority

1. All the way through this project and the development of the diagnostic kit compromises or adaptations have had to be introduced in order to adapt the lab-based assay for field use. Each modification introduced a further level of variability – these included multiple (10) versus single cores in the assay; use of unwashed cores (this was later reported to cause problems in measuring the absorbance. All the correlative graphs produced at Durham were derived using single core (half core) assays – precise, hand cut 0.5 cm cubes of tuber tissue cut with a razor blade were used originally. The cut surfaces of the cores were appreciably more ragged or irregular than the razor blade cut cubes. Exactly how much variation can be introduced at the different levels while still maintaining the lab correlation is difficult to answer but clearly in most if not all examples of the field trials this has been exceeded by the devised procedures.
2. Most of the absorbance data for the lab correlations were obtained using a spectrophotometer rather than the colorimeter used in the field trials. The change to a colorimeter was necessitated by the high cost of spectrophotometer. While spectrophotometers are inherently more sensitive than colorimeters for absorbance measurements there is no reason to believe that such sensitivity is required for the present colorimetric assay. Subsequent comparative work at Durham showed that the original WPA colorimeter and the new Hanna colorimeter gave readings which were directly comparable to the spectrophotometer values and were therefore adequate for the diagnostic kit within the range of absorbance values (See **Part 2** of the experimental **Section 3**).
3. The discovery that the tetrazolium dye / formazan product was unstable at alkaline pH values prevented the use of the enhanced blue colour change at the end of the SO reaction to provide a more sensitive assay. The adopted assay relied on the less sensitive pink > orange colour change for the quantitative measurements of SO generation. While this was easier it was also significantly less sensitive. Tests on the other water soluble tetrazolium dyes were disappointing and were either similar to XTT or much less sensitive in the diagnostic assays. WST's were much more expensive.
4. There were problems with the colorimeters used in the first field trials which led to variations in individual readings caused by imperfections in the plastic tubes used. Subsequently the tubes were changed from plastic to glass and then for the final field trials to optically correct glass cuvettes. The need for clean working practices when using the kit was regarded as a problem by several of the consortium members especially when large numbers of samples were required to be processed.
5. The measurement of bruising in potatoes is an inexact science. The process leading to pigment generation in tuber tissues is complex. Throughout the project this critical evaluation has been used in lab and a variety of field sites to test the validity of the correlation between bruise intensity and level of radical generation. Because bruise testing has long been the only way to assess susceptibility to mechanical damage the methods developed and used by the industry and different research groups vary enormously. To date in this project there does not seem to have been a single definitive method used for all investigations. Nor has there been true comparative studies to quantitatively compare results from different methods. All bruise assays are empirical and involve a significant element of subjective assessment which can introduce further levels of variation between samples. Lærke *et al* (2002) reported clear differences in bruise susceptibility depending on which method is used for the assessment – they used a pendulum-type impacter and a shaking platform device.
6. Examples of bruise damage assays carried out during this project on individual tubers exposed to single impacts showed a remarkably high level of variation as judged by the numerous scatter plots presented in reports and in consortium meetings. If this is a true reflection of the status of the tubers it would be extremely difficult to establish **any** correlation on this basis.
7. The subsequent observations by CUF that bruise intensity may actually change quite rapidly puts a whole new complexity on the use of secondary assay methods to assess bruise and mechanical susceptibility. Lærke *et al* (2002) reported that bruise susceptibility as measured by

two different methods, remained stable during storage. In this project we have assumed that this is the case for the duration between harvesting and testing (4-6 weeks). However in practice there are numerous examples where tubers are OK when put into store but subsequently become seriously susceptible during storage. It may be that there is a critical period between defoliation and harvesting that causes perturbations in the tuber bringing about increases in bruise susceptibility over the short term until the metabolism has stabilized in storage. Therefore it may not be possible to predict bruise susceptibility during the early period of harvesting or even within a period of the tubers being stored.

8. The work carried out with the different head radii produced some interesting results indicating that the shape of the impact head very much determines the amount of damage at the point of impact for a given force. A pointed impact head concentrates the energy of the impact into a small contact area while a flatter shaped head distributes the energy over a wider area and causes less damage. As a result the underlying damaged tissue volume may be very different ranging from a narrow column of very badly damaged cells to a wide column of comparatively undamaged cells. Depending on the properties of the tissues and the way that the impact energy is dispersed the columns may actually be differently shaped – barrel, inverted cone etc. Almost no work has been done on this aspect. However if the damage zone is too small or narrow (focused) then the core sampling becomes problematical. We know from our early experiments using single core samples that superoxide generation takes place within only a few cell layers of the surface of the tissue core. The smaller the diameter of the damage zone the greater the possibility that the sample core could miss the zone or at least for sampling a representative population of damaged cells. It would seem sensible to check this out especially since the impacters and the impact heads have undergone many changes from the original design.
9. Temperature effects on the reaction – this doesn't seem to be very temperature sensitive but being a chemical reaction it stands to reason that there will be some temperature effects. While the free radicals are highly reactive and their rapid reaction with the tetrazolium dye is likely to be relatively unaffected by temperature, the cellular detection of the impact, the resulting signalling responses and the production of the radicals are all enzymic in nature and therefore would be influenced in different ways by changing temperatures. Experiments to quantify this indicated that the effects were small within the temperature range likely to be encountered under field conditions (potato store, packroom, fields). Consequently the instruction manual for Blackspot Detect provides guidance on the recommended temperature range that the kit can be used ($10^{o}C$ – $20^{o}C$); outside this range the results could vary significantly.
   Temperature effects on the impacter have also been studied which show that with prolonged use, either with increasing ambient temperature or the temperature of the impacter increasing through being held for a long period, the force of impact does change. Almost certainly this is due to physical effects on the internal spring which provides the force. After correcting for external temperature variation the springs were found to perform almost identically (<0.5%). However on average a $+4^{o}C$ temperature difference caused a 6% increase in the spring force. This is difficult to quantify in a practical way to allow corrections for the end-user if that was thought necessary, but overall would influence any set of results in a non-linear way. In this respect it is useful to consider the effect of spring compression on the force delivered (**Section 3, Part 2.4** and **figure 2.7**).
10. The following relevant comments are taken from the commercial partners direct experience with the kit (See **Part 3.12**).
    McCains - The results between the bruise index and the damage index are disappointing; there was no correlation between the bruise index and the damage index. The reason for this needs to be determined before any future plans for the kit can go forward.
    Branston - The time to take and run the test is too long: If a field is approximately uniform in soil type, can we just not take one sample but with more tubers perhaps? Could we take one tuber from ten random spots across a field and treat as one sample? Can we shorten any of the steps?
    Greenvale -
    - Agronomists found the impactor and corer easy to use as a stand-alone piece of equipment.

- The time element was significant in reality they would find it easier to sample 50tubers impact in the field and hotbox overnight. As this needed less cleaning and lab time.
- Agronomists found maintaining timing and hygiene difficult during high pressure work periods
- Because we were still using the old colorimeter there was still variation in readings if the tube moved slightly.
- It is still possible that the impactor is exaggerating the susceptibility

Initially samples tested by the kit did seem to mirror results from the hot box but this appears to be where the test showed susceptible the hot box developed a very high index indeed. Others showed very little correlation between the two tests. In all cases the prediction of bruising susceptibility was not reflected in the harvested samples. It became apparent that the impactor was possibly bruising the potatoes too much and that the impact did not reflect actual harvesting conditions. This was the first year we let the agronomists have ago themselves and as a result only 10 out of 20 fields were done as they found the kit time consuming to put into practice. They have also not completely followed through consistently with the follow up information. What is apparent though, is where bruising indexes were recorded by factory QCs they are significantly below the indicated indices when using the impactor. One sample had to be aborted due to contamination of soil/skin material after a poor washing. This is a vital area of control that can be difficult to do without the right room and facilities.

11. Overall this has been a very challenging project where we have tried to translate an established biochemical principle based on lab precise manipulations to an applied problem in the potato industry. The variability encountered in the field environment and in the compromises necessary to adapt the chemistry and hardware for field use has led to results which are at best no better than existing methods and at worse provide no trustworthy indication of the bruise status of the tested crops. On the positive side many novel and useful results and observations have come from the work both from the field trials and the lab-based work which should prove useful in the future. Many of the observations should be followed up to test the conclusions arrived at from the work. The hardware has proved to be both useful and easy to use and it is hoped that these items will be further developed into products that the Industry will adopts as standard tools.

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