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VI1240 - Assessment of heat for sterilisation of plant material

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A short report prepared for

ZESPRI International Ltd

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This report has been prepared by The New Zealand Institute for Plant & Food Research Limited (Plant & Food Research), which has its Head Office at 120 Mt Albert Rd, Mt Albert, Auckland.

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Aim

Develop and implement an assay to asses if kiwifruit cambium tissue is living or dead, and apply this to kiwifruit tissue which has been heat treated.

Introduction

Plant tissue is heat sensitive. All physiological systems have an optimum temperature. Above the optimum temperature function declines and as the temperature continues to increase function will stop, usually irreversibly, generally resulting for protein degradation. The purpose of this work is to determine how high a temperature kiwifruit canes can be heated, and for how long, before the cambium tissue is damaged. Not only is there a temperature response, but also the duration at the high temperature which determines the fate of the tissue.

In this work kiwifruit canes were heated to a controlled temperature and held at this temperature for various lengths of time. The cambium tissue was then examined to see if it was still viable (living). This was determined by observing active membrane function and active enzyme function within the symplast.

The assay used is based upon uptake and symplasmic cleavage of fluorescein diacetate (FDA) by cytoplasmic esterase's to produce a fluorescein that can be observed by its fluorescence under blue light. This is the currently accepted method to test whether tissue is alive or dead (e.g. Krasnow et al. 2008). Accumulation of FDA requires a functioning membrane and cleavage requires active esterase's. FDA has been used to study cell viability in a wide range of systems, including mammalian cells, algal cells, plant cell cultures, protoplasts, embryos and seeds (see Krasnow et al 2008 for references).

It needs to be noted that the FDA assay used was used on the cambium tissue of a cane as well as a kiwifruit trunk, so is measuring the viability of this tissue. The assay was not applied to dormant buds, so does not give any information of whether the bud is damaged by various temperature treatments. This approach was taken as the question from ZESPRI was aimed at what temperature treatments a kiwifruit trunk could sustain, and buds are not involved in this system.

Assay

A stock solution of 2% FDA in acetone was made up and kept at -20 ˚C. All tissue samples were washed in stained at room temperature in a 50mM phosphate buffer pH 5.5 containing 1mM $CaCl_2$.

Heat treatment involved heating segments of cane in a temperature controlled water bath.

Segments were first cut and placed in tap water ~16 ˚C and given 0, 2, 5, 10, 20, 30, or 60 minutes in a temperature controlled water bath. At the end of the heating treatment segments were cooled in tap water (~16˚C) for several minutes.

The first 5mm at the end of a heat treated segment was discarded, and thick hand sections (~0.1mm) of kiwifruit cane were made by hand and collected in the phosphate buffer until samples from all treatments were available. The longest treatments samples were washed in the buffer for about 10mins, and the shorter treatments for longer. Some of these washed slivers were then added to 1ml buffer +10µl of FDA stock for staining, and some kept back in the buffer without stain as controls. At least 20 minutes of staining was carried out, and then the stained samples transferred into buffer for a wash.

With trunk samples, a scallop of the trunk was cut containing both bark and inner wood and kept in a plastic bag to reduce water loss during transport to the lab. Samples were prepared by shaving off ~2mm of the outer edge and then cutting thick samples as controls and for staining, using the same procedure as for the cane.

At least four controls (ie no stain) and four FDA stained samples from each temperature duration were then inspected under a fluorescence microscope and photos taken.

Most of the work was done using canes taken from Bruno seedlings, as when this work started the Hayward vines were about to be pruned and pruning of the Bruno block was delayed for me to compete this project. There is no reason to believe that there will be a marked difference in heat response between kiwifruit cultivars so this was not seen as a problem.

Bud burst

Once a potential temperature treatment was found that did not entirely kill the Bruno wood tissue, cuttings were put through a series of time treatments and their ability to break bud determined, as a means to assess whether the bud was more or less sensitive to a temperature treatment than the cambium of the stem.

Twenty cuttings with just one bud were taken and immediately put through each of the time sequence of 0, 2, 5, 10, 20, 30 and 60 min at 50˚C wit the cutting completely immersed in the temperature controlled water. Also, another 20 cuttings were treated for 60 min. with the bud kept out of the water by means of a small polystyrene block.

Results

The cambium area of cane and trunk tissues was easily identified, though the FDA stain was not essential to do this as there was a lot of natural fluorescence from this region. Samples that had been heat treated for a long period, or shorter periods at high temperature clearly showed no natural fluorescence, nor fluorescence with FDA staining.

Visual image 30min | Control fluoresence | Heat treated 90 ˚C

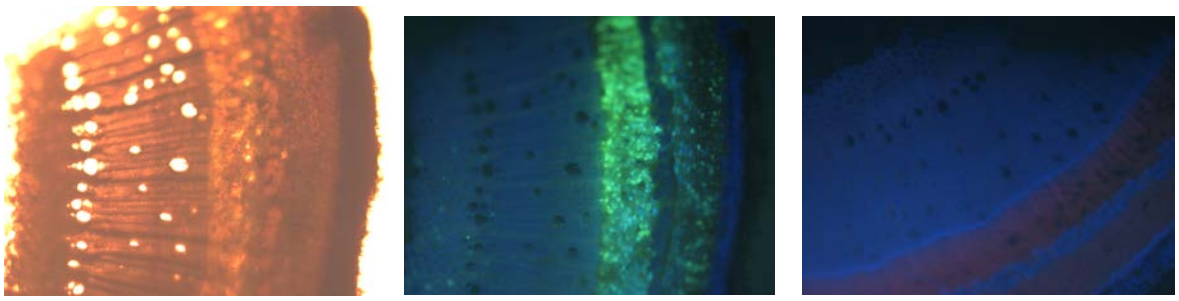


Figure 1. Heat treatment of Bruno cane 1-7-2011, showing the visual image, the fluorescence image after staining with FDA, and the fluorescence image obtained after heat treatment and then staining with FDA

Figure 1 shows the visual image in which the anatomical details are clear – xylem vessels being the ‘holes’ with ray cells visible as the dark lines crossing from the cambial ring into the wood. The bark appears as a dark outer ring with a circular small cell structure below the bark. In the fluorescence control the cambium is glowing greenish-blue with a dark blue line separating this

into two zones. With careful inspection fluorescing ray cells can be seen extending from the cambium into the xylem. The split of the cambium was clear in most of the stained images and I expect it was caused by the separation of the cambium during cutting of the sections. The bark 'naturally' peels off taking the phloem with it at the cambium. The heat treated sample shows no fluorescence at all, the blue image being from the illuminating blue excitation light. There is a clear red fluorescence from the cambium region – chlorophyll fluoresces in the red. In a small number of samples there was a red fluorescing image of algae on the outside of the back zone, with the stem hairs being blue as the xylem region. This is an extreme treatment with extreme difference between the control and heat treated tissue. With lower temperatures and shorter treatment times the difference was not so clear.

With intermediate treatments, heat treatment reduced both the natural and FDA derived fluorescence.

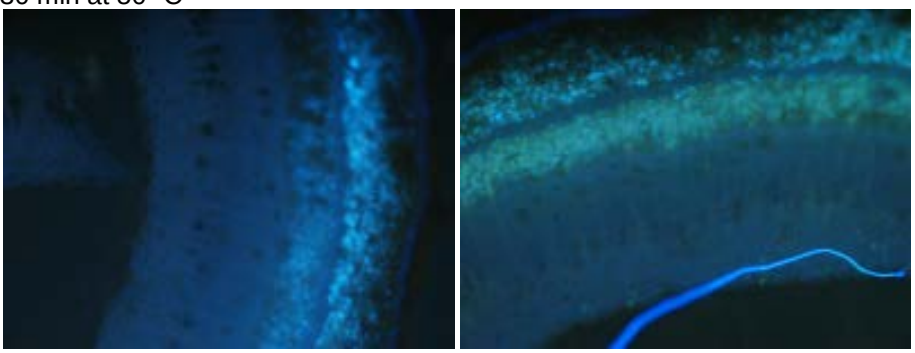
Typical photos for each time duration, with a specific temperature, of the control and FDA stained samples were selected and printed. This time sequence of heat treatment was then assessed for what time duration resulted in a major loss of fluorescence in the cambium region (see Figure 2 for an example).

Table1. Temperature, and duration at this temperature at which cell viability was lost

Date	temperature ˚C	duration to lose most fluoresence (mins)	cultivar
20/07/2011	50	30/60	Bruno
26/07/2011	50	60	Bruno
28/07/2011	50	60	Bruno
26/07/2011	50	30/60	Hayward
21/07/2011	55	5/10	Bruno
22/07/2011	55	5/10	Bruno
14/07/2011	60	2/5	Bruno

At 50 ˚C the cambium appeared to be still viable after 30 minutes, and in 2 of the 4 trails at 60 minutes. Higher temperature was dramatically more damaging, with 60 ˚C killing the cambial cells after just 5 minutes.

30 min at 50^{o} C



60 min at 50^{o} C

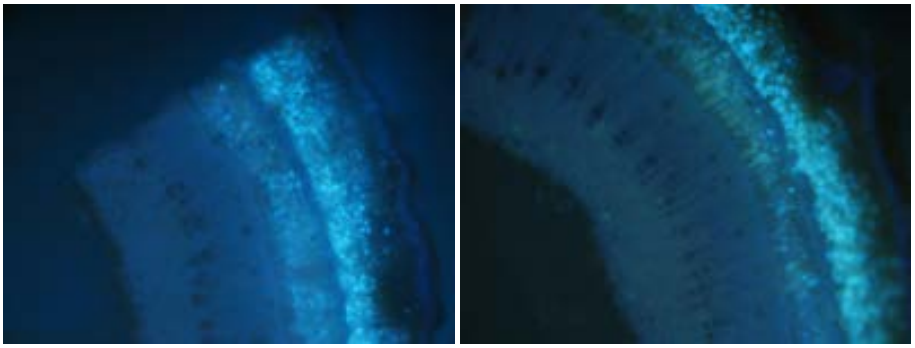


Figure 2. 50˚C heat treatment showing the transition from what is assessed as viable to probably not viable. Note the reduced fluorescence in the 60minute treatment compared to the 30 minute treatment.

The trunk tissue had been treated in the field by a hot-air system organised by Shane Max, and bark/wood samples were given to me to test with the FDA approach. The heat treated trunks samples showed considerable blackening, probably from cellular damage associated with release of phenolics. On removing the outer tissues, to take samples for FDA analysis, samples that were black all indicated non-viable tissues, while wood samples that were still white were viable according to the FDA assay. The FDA analysis gave no further information than the visual blackening of the tissue.

Bud burst

Bruno cuttings

Treatment Time immersed in water at 50^0C	Total number of cuttings	Total number of cuttings that budded
0 minutes	20	6
2minutes	20	20
5 minutes	20	20
10 minutes	20	18
20 minutes	20	15
30 minutes	20	12
60 minutes fully immersed	20	0
60 minutes partially immersed	20	12

Each treatment involved 20 cuttings. Only 6 of the control (no heating) cuttings burst bud while many more did with a short heat treatment. With a 60 min heat treatment none of the totally immersed cutting burst bud, but 12 of those that had the bud held above the water did. A short heat treatment has clearly greatly enhanced bud burst.

Conclusions

The cambium of Bruno can probably survive 60 minutes at 50˚ C, and certainly 30 minutes. Five degrees hotter greatly reduced this time taken to result in significant damage as indicated by FDA fluorescence.

This work was done looking at cambium viability, which might be different to that of a bud. On completion of the stem heat treatment measurements, Bruno cuttings, with 1 bud/cutting were give the same 50˚C heat treatment, and then placed in water in a controlled temperature room with lights by day to look at bud burst. A second set of cuttings were treated for just 30 minutes at 50˚C with the buds out of the hot water, to look at whether this heat treatment did affect the buds differently to the treatment when the entire cutting (including bud) was heat treated.

Results are not yet available for the cutting trial. The first of these buds are breaking. This report will be updated once this data is available, and forwarded to ZESPRI by the end of September, as per the contract.

Also, under KRIP funding I am proposing to look at phloem function at different cane temperatures. Using radio labelled 14˚C-photosynthate movement along a cane as an in vivo measure of phloem function, a length of cane will be heated to a well controlled temperature by passing temperature controlled water over this segment, while continuing to follow phloem function. This will give a measurement of phloem function at various temperatures. Previous unpublished work has shown that phloem transport through bean stem was unaffected at 54˚C but at 56˚C it was completely inhibited.

A temperature treatment of 50˚C was chosen to test bud burst as the FDA work indicated that this temperature did not entirely kill the cambium tissue. We found that using this temperature that bud burst was enhanced, compared to no treatment, with up to 30 mins total immersion, while with 60 mins total immersion there was zero bud burst. But, 60 mins at 50˚C with the bud kept out of the heated water resulted in bud burst comparable to 30 min with complete immersion. It appears as if the bud is more sensitive to the temperature treatment than is the cambium tissue.

So in summary, a heat treatment at 50˚C for up to 20-30 min appears to not markedly inhibit bud burst of a cutting. It the bud were not immersed in the water a longer treatment would be possible.

Acknowledgements

Drs Ian Hallett gave advice early on in this work as how best to label with FDA and how to assess the fluorescence images. Dr Andrew Allan gave very helpful advice on which assay to use and references to current work based on this method. This help was important in ensuring that I was heading in the right direction and gave me increased confidence in the approach which I had read about but had no experience in using .

References

Krasnow M, Mathews M, Shackel K. 2008. Evidence for substantial maintenance of membrane integrity and cell viability in normal developing grape (*Vitis vinifera* L.) berries throughout development. Journal of Experimental Botany **59**, 849-859.