Gibberellin and cytokinin in synergy for a rapid nodal multiplication system of avocado

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SUMMARY

Conventional clonal avocado rootstock propagation relies on a 40 year old method and derivatives thereof that is labour intensive and does not facilitate cost effective supply of clonal rootstocks for the rapidly developing needs of the industry. This is especially relevant if high density plantings or clonal rootstocks are to be adopted. An alternative tissue-culture-based clonal propagation system could provide efficient, cost effective, high volume and sterile production of clonal rootstocks.

Avocado, being a woody plant species, is not highly amenable to tissue culture, with most attempts confined to nodal culture methods. Thus far, nodal cultures of avocado show very slow growth and produce stunted shoots, limiting rapid shoot multiplication.

The current study aims to develop a system to multiply nodal avocado shoots using gibberellins and cytokinins to produce taller shoots with a higher number of nodes for continuous culturing. Cytokinins, the most important plant growth regulator in shoot regeneration, and gibberellins, which promote internode elongation, can synergistically improve the quality shoots for rapid nodal multiplication. The effect of different combinations of the natural aromatic cytokinin, *Meta*-topolin, and the active gibberellin, GA3, on bud breaking time, shoot height, number of open leaves, overall shoot quality, vitrification and callus production in continuous culture of avocado nodes were assessed. The results reveal optimal hormone combinations for improved nodal multiplication for the avocado tissue culture pipeline.

Annotations: MT – *Meta*-topolin, GA3 – Gibberellic acid.

INTRODUCTION

Avocado (*Persea americana* Mill) is a grafted crop comprising a fruiting scion (most commonly cv. Hass) grafted to a rootstock cultivar with beneficial field characteristics. These rootstocks can be propagated by seed or by clonal propagation, which requires root-induction on budwood cuttings. Seed-propagated rootstocks suffer from high genetic heterogeneity and thus poor on-field consistency due to the out-crossing nature of avocado (Ayala Silva & Ledesma, 2014) (Barceló-Muñoz, Encina, Simón-Pérez, & Pliego-Alfaro, 1999)

ds (Barceló-Muñoz, Encina, Simón-Pérez, & Pliego-Alfaro, 1999). On the other hand, clonally propagated rootstocks are true to type, and can be selected for improved yield characteristics and abiotic and biotic stress resistances, which ultimately decide the return on investment.

The current industry practice of generating clonal rootstocks employs an arduous double grafting technique, where budwood from a rootstock mother plant is first grafted to a seedling nurse plant. Once the graft union has taken, the plant is then etiolated and auxin treated at the base of the scion stem as a prerequisite for adventitious root induction. Successfully rooted scions are then grafted with the desired fruiting scion and removed from the nurse seedling. This process of clonal propagation is lengthy and expensive. As such, although clonally propagated rootstocks may provide higher and more consistent yields, their increased price and limited availability is prohibitive to large-scale industry uptake.

Tissue culture has the potential be a very effective and efficient alternative for clonal rootstock production of avocado. However, avocado has performed poorly in a tissue culture environment, similarly to other woody plant species (Bairu & Kane, 2011). Many attempts have been made to establish industry applicable micropropagation systems using juvenile and mature avocado. Even though positive results were obtained when juvenile material was used, *in vitro* culture of physiologically mature material is still problematic (Barceló-Muñoz *et al.,* 1999). Several problems, such as poor elongation and high rates of defoliation, tip die-back, vitrification and stunting have been observed.

This study evaluated the combined use of cytokinin and giberellin to improve avocado shoot elongation and quality in culture; both prerequisites for a nodal multiplication system for continuous culture. *Meta*-topolin , the most active of the natural aromatic cytokinins, was used instead of the widely applied 6-benzyleamion purine (BAP), with the aim to eliminate high callusing, necrosis and negative effects at the rooting stage (Gentile *et al.,* 2014). Giberellic acid is known for its promotive effects on internode extension, leaf growth and release of apical dominance (Gonbad, Sinniah, Abdul Aziz, & Mohamad, 2014). By utilising synergistic effects of GA3 and MT in avocado culture, the objective of this study was to optimise a protocol for shoot regeneration in order to develop a rapid clonal multiplication system for avocado.

METHODS

Plant material

Budwood of cv. velvick was collected from grafted plants maintained in a glasshouse at 26 0C under natural light conditions at The University of Queensland Australia. Twigs of 50 - 60 cm were collected and all leaves removed before sterilisation.

Surface disinfestation

Budwood twigs were washed under running tap water for 45 minutes and transferred to a laminar flow cabinet. Under aseptic conditions these were then washed with 70 % (v:v) ethanol for 3 minutes followed by rinsing with sterile distilled water three times. The twigs were then immersed in 3% (v:v) bleach solution (Ajax Finechem) with three drops of tween-20 (Sigma Aldrich, Australia) for three minutes. Finally these were washed several times with sterile distilled water to remove any traces of bleach and surfactant.

Initiation

Initiation medium comprised ½ strength Lloyd and McCown's (1981) Woody Plant Medium (WPM)(Phytotechnology laboratories) with 20 gL-1 sucrose and 2.5 gL^{-1} phytagel (pH 5.65); autoclaved and stored for 24 h before explant inoculation. Under aseptic conditions the surface disinfested twigs were cut into ~1.5 cm single node sections each containing an axillary bud. Shoot tips were excluded to avoid any variation in inherent auxin levels and physiology. Cultures were incubated in a growth room at 25 °C with a 16 h photoperiod for seven days to initiate and carefully screen for fungal or bacterial contamination.

Shoot regeneration

Shoot regeneration media was composed of full strength WPM with 2 mgL^{-1} $AgNO_3$, 20 gL^{-1} sucrose, 2.5 gL^{-1} phytagel and the treatment hormones detailed in Table 1. Control treatments with no hormones or with each individual hormone in the media were also included. Magenta GA-7 plant tissue culture tubs were used in this phase and pH was set to 5.65 using 1 M NaOH before autoclaving. Twenty clean nodal sections were established in each of the ten treatment media and incubated in a growth chamber as above. In order to maintain proper supply of nutrients and test hormones, cultures were transferred to respective fresh media every four weeks. At each transfer a clean cut was made at the base of the node to ensure continued nutrient absorbance.

Table I – Hormone treatments at shoot regeneration

Hormone Treatment	MT concentration mgL^{-1}	GA_3 concentration mgL^{-1}
Treatment 1	-	-
Treatment 2	0.1	-
Treatment 3	1.0	-
Treatment 4	-	0.1
Treatment 5	-	1.0
Treatment 6	0.1	0.1
Treatment 7	0.2	0.2
Treatment 8	0.3	0.3
Treatment 9	1	0.1
Treatment 10	2	0.1

Measurements and data analysis

Observations for bud breaking were made at day 8 after establishment. Measurements of shoot height, number of open leaves and shoot quality (0 – 5 scale, considerations – vitrification, chlorosis, necrosis, healthy leaves) were recorded at day 15, 30 and 45 after establishment on shoot regeneration media. Other observations for verification, chlorosis, callus production, shoot tip die back, defoliation and necrosis were also recorded. Descriptive statistics were generated and analysis of variance was computed at confidence level 0.05 using the statistical software IBM SPSS 23.

RESULTS AND DISCUSSION

Clear differences in the shoot habit with respect to bud-breaking, shoot tip die back, defoliation and necrosis were observed in nodal explants grown on hormone containing media compared to no hormone controls. No bud breaking was observed at day 8 in non-hormone medium, while all other treatments containing MT or GA3, or both, showed various levels of bud breaking (data not shown). There was 100% bud breaking when 0.1 mgL-1 MT alone or

combination with GA3 was present. At day 30, shoot tip die back, defoliation and necrosis were observed for hormone-free cultures, while shoots maintained on hormones remained green and healthy (Figure 1). Furthermore, by day 45, ~65% of hormone-free cultures were suffering from senescence.

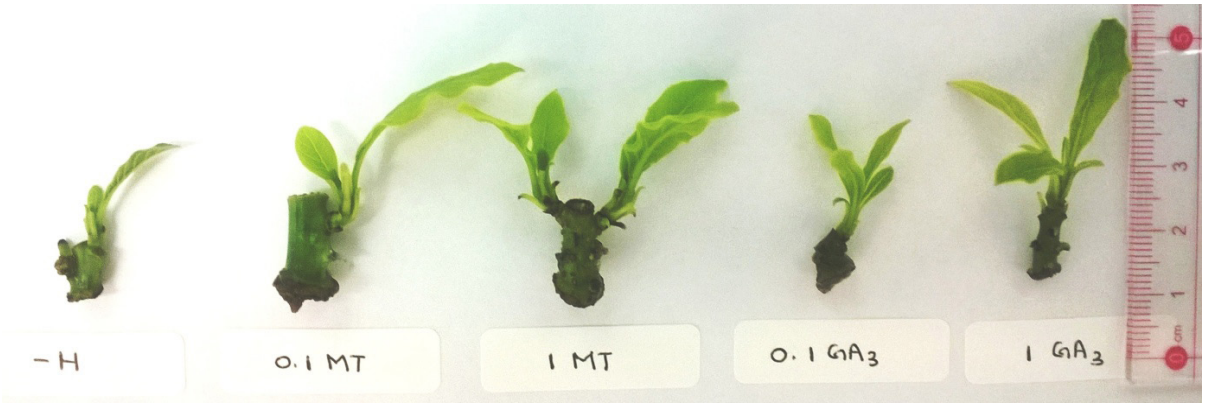

Figure 1: Representative shoots after 30 days in various regeneration media. (Concentrations of Meta-topolin (MT) and gibberllic acid (GA3) are shown in mgL-1, -H: No hormone control)

To date there is no reported use of *Meta*-topolin as the source of cytokinin in avocado tissue culture, despite it proving very effective in shoot regeneration of other woody plant species including pine (De Diego, Montalbán, & Moncaleán, 2010) and sweet orange (Niedz & Evens, 2011). While in general, hormone treatment improved explant shoot growth, a clear difference in the shoot habit was observed under different hormone regimes (Figure 1 & 2). GA3 in the media promoted shoot growth relative to no-hormone controls, however at a high concentration (1 mg L-1), this resulted in verification and very thin elongated leaves relative to MT-treated shoots (Fig. 1). On the other hand, *Meta*-topolin could effectively eliminate common problems of shoot tip die back and defoliation due to hormone stress. This is consistent with the results in other species, including that of Mirabbasi & Hosseinpour (2014), who saw reduced of shoot tip necrosis and prevention of abnormal growth using low levels of *Meta*-topolin in *Ulmus glabra*. Compared to individual hormone or no hormone treatments, combined hormone treatments resulted in broader, healthier leaves (Fig 1 & 2).

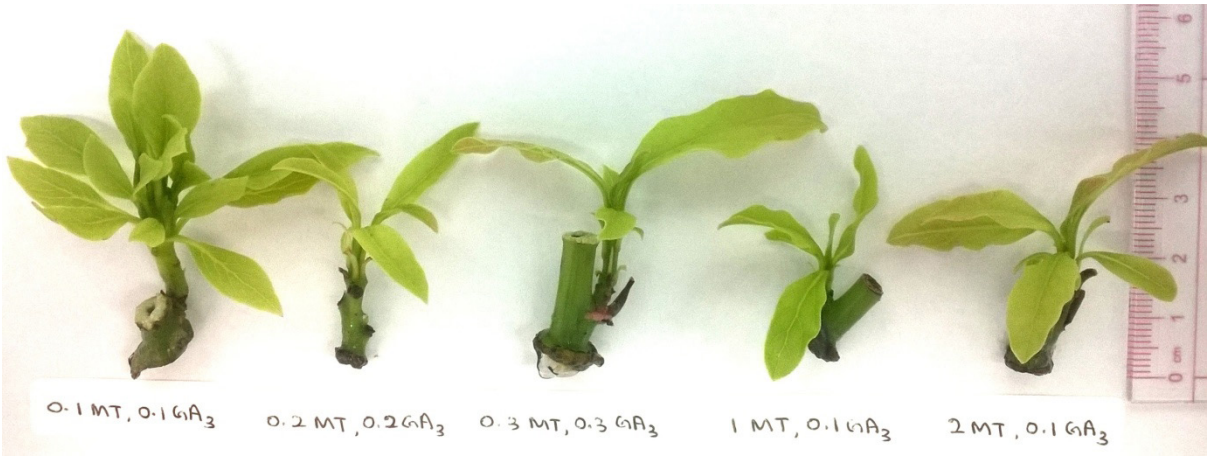

Figure 2: Shoots in regeneration media with both MT + GA3 after 30 days. Hormone levels are shown as mgL-1

Incorporation of cytokinins with either auxin or giberellin in shoot regeneration media is common practice in tissue culture. Ahmed *et al.* (2001) used cytokinin, auxin and GA3 for juvenile avocado shoot regeneration and found 1 mgL-1, 0.1 mgL-1 and 0.1 mgL-1 respectively to be the most effective among treatment groups. This agrees with the positive effect of 0.1 mgL-1 GA3 seen here. In most instances cytokinins are coupled with auxins, to deliver desired results. Our approach coupling cytokinin with GA3 has delivered very promising results, producing taller shoots with higher number of nodes that can be segmented and used as mother plants for next sub culturing steps.

In an investigation by Castro *et al.* (1995), also using mature cv. velvick, axillary buds failed to regenerate in WPM containing high levels of BA and GA3 (both 2 mgL-1). Here, we observed a negative effect on shoot quality using high levels of GA3, but no prevention of bud regeneration. Moreover, combining 2 mgL-1 MT with a low concentration of 0.1 mgL-1 GA3, gave 100% shoot regeneration. This difference may therefore be due to the replacement of BA with MT and the greatly reduced GA3 levels in the present study. However, our data was consistent with the results of Vega (1989), who also showed100% (maturity of material not specified) shoot regeneration using the same concentration and combination of Castro *et al* (1995) in ½ strength Murashige and Skoog (1962) basal medium.

A significant difference ($P < 0.05$) between treatment media for shoot height at day 15, 30 and 45 was noted. By day 45, treatment 6 (0.1 mgL^{-1} MT + 0.1 mgL^{-1} GA_3) produced significantly taller shoots, indicating a clear synergistic effect of these hormones on shoot height. In regards to shoot quality, no significant difference was noted between the various treatment groups involving combined hormones.

CONCLUSIONS

Meta-topolin and GA_3 at the levels applied in the present experiment helped to prevent necrosis, defoliation and shoot die back. A synergistic effect of MT and GA_3 was noted for promotion of shoot height, number of open leaves and overall quality of the shoots. An optimum combination was identified for supporting excellent shoot health, with potential to develop a rapid nodal multiplication system for cv. velvick. This takes us one step closer to establishing an industry applicable solution for micropropagation of avocado rootstocks.

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Factors affecting avocado shoot health in culture

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Avocado tissue culture systems have the potential to provide a sterile, high-throughput, cost efficient alternative for clonal avocado propagation. Tissue-culture potentially allows multiplication of clonal stocks to provide much more cost efficient, uniform, reliably high-yielding plants that are guaranteed disease-free. However, clonal propagation of avocado in culture is complicated by high inter-cultivar variability in explant health in culture.

We are investigating avocado culture media for shooting of nodal explants from mature trees of a number of varieties. Numerous variables were found to affect shoot growth and survivability, and the effect of carbohydrates in the media will be discussed here. We are also examining endogenous carbohydrate profiles in explants harvested for tissue culture. The effect of etiolation on these profiles, and also on the carbohydrate profiles in budwood grafted for clonal propagation using the traditional Brokaw method (patent #4012866, Ernst 1999) are being determined. Carbohydrates are essential for energy and developmental signalling in plants and we aim to relate our results to plant growth rates and propagation.

Key Words: Avocado, Tissue culture, Shoot health, Sugars/carbohydrates, Propagation.

INTRODUCTION

Current industry practises for avocado plant multiplication are soil based techniques. Whilst many large-scale avocado nurseries internationally are based on these techniques, they are value-constrained by the large space requirement, long propagation time, in addition to soil-related quarantine and trade issues. To resolve these constraints, a tissue culture-based multiplication program for avocado plants is highly appealing.

In vitro plant tissue culture is the aseptic culture of tissues under controlled nutritional and environmental conditions to produce clonal plants (Hussain et al. 2012; Smith, MK & Drew, RA 1990). However, there is still a pressing need for extensive work in the field of basic tissue culture methods for many crop plants, including avocado (Smith, M & Drew, R 1990). A number of studies report optimization of tissue-culture for a number of woody species, including avocado, but results cannot always be transferred between species, or even between cultivars. Major impediments to tissue-culture of avocado remain including 1) inter-varietal inconsistency and poor shoot proliferation of mature explants in culture and 2) poor induction of roots on successfully cultured mature shoots (Pliego-Alfaro, López-Encina & Barceló-Muñoz 1987). This is a huge bottleneck for clonal avocado propagation, as elite rootstock material currently exists only in orchard-based repositories of mature trees.

Sugars produced during photosynthesis provide the cellular energy for plant growth and development and are essential signalling molecules affecting body-plan patterning (Lastdrager *et al*., 2014). In tissue culture, photosynthetic function is drastically reduced (Zulfiqar *et al*. 2009a). Therefore, a continuous supply of exogenous carbohydrates in culture is essential for high energy requiring processes such as shoot and root initiation (Custódio, Martins-Loução & Romano 2004; de Paiva Neto & Otoni 2003). Sucrose (12-Carbon) is the most abundant native carbohydrate in the photosynthate of most angiosperms (Zimmermann & Zieglar 1975). It shows efficient uptake across the plasma membrane and has successfully supported shoot proliferation and root induction in woody species including in peach (Prunus persica) (Ahmad, Touqeer et al. 2007) and apple (Malus domestica) (Yaseen *et al*. 2009). As such, sucrose has been the carbohydrate of choice for micropropagation of woody species to date (Romano, Noronha & Martins-Lou o 1995; Swamy, M. K., Balasubramanya & Anuradha 2010). Nonetheless, Sorbitol, mannitol, fructose, glucose, and galactose are additional sugars, based on a 6-Carbon (C6) unit structure, that have been extensively studied in a number of woody plant such as pear (Kadota, Masanori & Niimi 2004) and apricot (Marino *et al*. 1993).

The effect of carbon sources other than sucrose, sorbitol and glucose on shoot proliferation remains unknown for avocado (Kadota, M, Imizu & Hirano 2001). Given that the effect of carbon source on growth in culture often appears to be related to the species used, in the present work, the effect of exogenous carbon source on avocado explant health and shoot growth in tissue culture was examined. The ultimate aim is to optimise a media formulation for clonal avocado propagation of multiple varieties.

METHODS

Plant material

Stem cuttings from mature avocado plants of two industry relevant rootstock cultivars in Australia, Velvick and Kidd, were taken from plants sourced from Anderson's Horticulture Pty Ltd (Duranbah, New South Wales) and maintained in glasshouse conditions at the University of Queensland (natural day length; 26^{0}C). Plants were derived from grafted bud-wood taken from mature field trees (>10 years old) and grown as grafts in the glasshouse.

Sterilisation

Stem cuttings approximately 20-25 cm long, with multiple bud nodes were disinfected with liquid handsoap (Microshield, Johnson&Johnson) for one minute and washed under running tap water for 45 minutes. They were then washed with 70% alcohol (v/v) for three minutes followed by three

washings of sterile distilled water. The shoots were then soaked in 5% sodium hypochlorite solution (v/v) (Ajax Finechem, Thermo Fisher Scientific) containing 3-4 drops of Tween 20 (Sigma) for four minutes. They were then thoroughly rinsed six times with sterile distilled water.

Initiation

Under aseptic conditions, surface sterilised stems were divided with a sterile scalpel into numerous 1-1.5 cm nodal sections, each containing a single axillary bud. Immediately after cutting, the basal end of each section was placed into solid initiation media comprising Woody Plant Media, WPM (McGowan and Llyod 1981), 2% sucrose and 8g/L agar. These were grown at 25 °C (16 hour light days) in a controlled growth room for two weeks to ensure elimination of contaminants.

Shooting Media Treatments

To test the effect of carbon source on growth of mature shoots, sugars and sugar alcohols of various Carbon chain lengths were supplemented in shooting media. These were glycerol (C3), erythritol (C4) and xylose (C5), at three different concentrations (1%, 2% and 4%). Sucrose, sorbitol, and fructose had previously been examined for both cv. Velvick and cv. Kidd (J. Hiti Bandaralage pers. com.), with 2% sucrose and 2% sorbitol supporting best growth for cv. Velvick and cv. Kidd respectively. As such these were chosen as the controls. The sugars were added to a basal WPM (McGowan and Llyod 1981) medium. Each carbon source was tested on 10 replicate mature nodal cuttings each for cv. Velvick and cv. Kidd. Cultures were grown as above for 8 weeks, and sub cultured at 4 weeks to replenish degraded nutrients. Cuttings were characterised morphologically every two weeks for shoot length (mm), overall health (subjective scale 0-5 based on necrosis, browning, chlorosis vitrification and leaf drop) and leaf expansion.

Statistical Analysis

All data generated from this study were evaluated using one-way Anova in Microsoft Excel (2010) at 5% significance.

RESULTS

In order to determine the relationship between carbon source and avocado shoot health in culture, mature avocado nodal stem sections were cultured on a number of sugars, or sugar alcohols, of different carbon-chain lengths and concentrations. Effects were analysed on two industry relevant rootstock cultivars: cv. Velvick and cv. Kidd.

For mature cv. Velvick, excellent shoot growth was recorded on 2% sucrose and 2% xylose (Figure 1). Explants appeared to have poorer shoot elongation on the sugar-alcohol control (2% sorbitol) compared to sugar control (2% sucrose), however this difference was statistically insignificant ($P > 0.1$). Furthermore, shoots on both 1% and 2% glycerol were significantly inferior at supporting shoot elongation than the sucrose control ($P < 0.01$; Figure 1). Explants on 4% glycerol were smaller and of poorer health than those on sucrose, with 60% of explants dying (Figure 1). However, of the surviving explants, the shoot length on 4% glycerol was not significantly different to the sucrose control ($P > 0.05$). All explants on xylose had slightly poorer shoot elongation than the sucrose control, particularly for 4% xylose (40% growth reduction); however the differences were not significant for any groups (all $P > 0.2$). None of the erythritol-supplemented explants survived the shooting experiment.

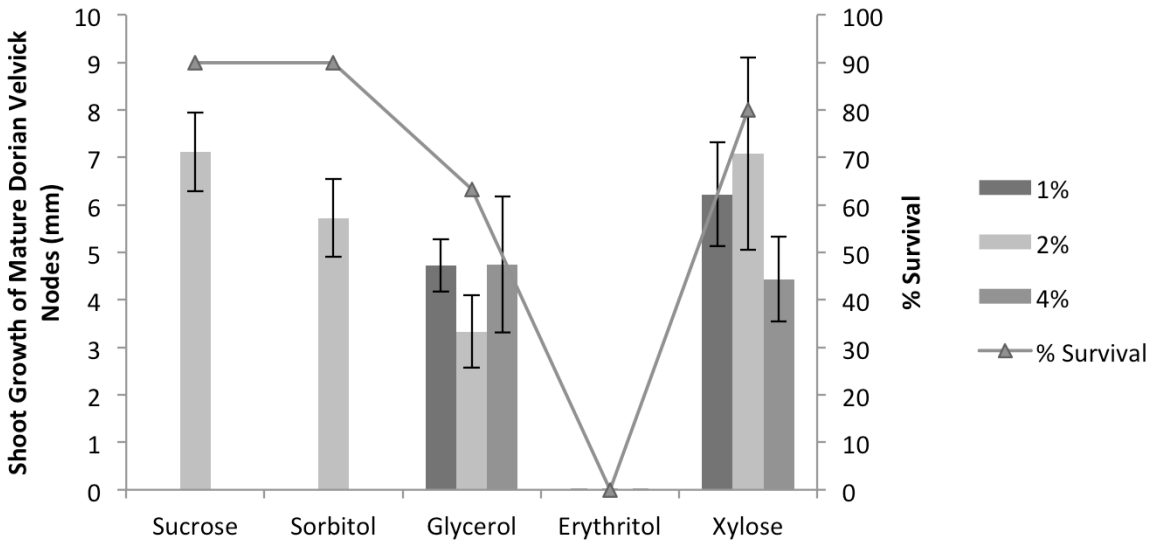

Figure 1: Mean shoot growth of mature avocado cv. Velvick explants after seven weeks (bar graphs) and the percentage survival (line graph). Carbon sources were tested at 1%, 2% and 4%, relative to 2% sucrose and sorbitol controls. Data are means +/- SE (n= 10).

The effects of different types and concentrations of carbon source on shoot induction were found to be highly cultivar dependent. For mature nodal explants of cv. Kidd, 1% glycerol and 4% xylose showed a similar shoot elongation of ~4mm, which was statistically greater ($0.002 < P < 0.009$) than the 2% sucrose control (Figure 2). The sugar-alcohol control sorbitol (2%) also supported better shoot elongation and survival than sucrose, with 50% survival versus 20% survival for sucrose (Figure 2). Moreover, sorbitol produced the healthiest shoots across all visual parameters including necrosis, leaf-drop, chlorosis and vitrification (data not shown). These results contrast the relatively poor growth of cv. Velvick shoots on sorbitol, glycerol and xylose when compared to sucrose. Similarly to cv. Velvick however, cv. Kidd did not survive on erythritol-containing medium, with shoot death observed as early as week 3 (Figure 4).

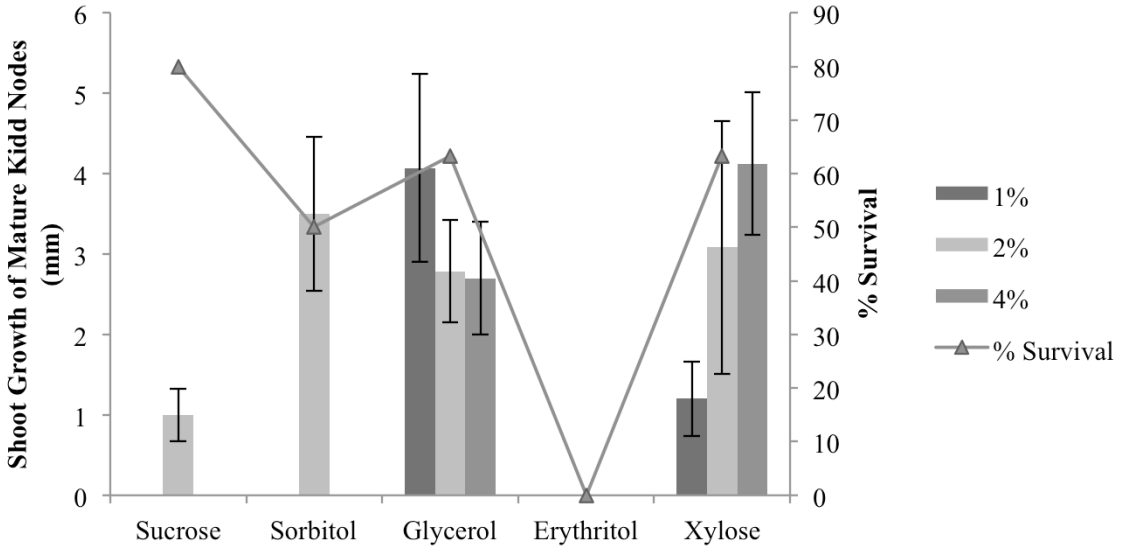

Figure 2: Mean shoot growth of mature avocado cv. Kidd explants after seven weeks (bar graphs) and the percentage survival (line graph). Carbon sources were tested at 1%, 2% and 4%, relative to 2% sucrose and sorbitol controls. Data are means +/- SE (n= 10).

Although mature cv. Velvick shoots showed poor growth on sorbitol relative to sucrose, cv. Velvick generally outperformed cv. Kidd explants on all sugars, including sorbitol. Indeed, the differences of performance between cv. Velvick and cv. Kidd were most apparent when the best performing carbon sources for both were compared; 2% sucrose and 2% sorbitol, respectively (Table 1). This difference was most apparent for the 2% sucrose control.

Table 1: Comparison between mature cv. Velvick and mature cv. Kidd nodes on sucrose and sorbitol supplemented media

Carbon source	Cultivar	Average shoot growth (mm) ± SD	p-value	Survival (%)
2% sucrose	Velvick	7.11 ± 2.60	4.41E06**	90
	Kidd	1.0 ± 0.93		80
2% sorbitol	Velvick	5.72 ± 2.46	0.058858	90
	Kidd	3.5 ± 2.15		50

DISCUSSION AND CONCLUSIONS

Carbohydrate supplementation is essential for the health and proliferation of explants in tissue culture. In the present work, the effect of exogenous carbon source on avocado explant health and shoot growth in tissue culture was examined, with the aim to optimise existing protocols.

The carbon source in the culture media resulted in significant differences with regards to shoot elongation, leaf expansion and survival of nodal explants from cv. Velvick and cv. Kidd, two industry-relevant rootstocks in Australia (Figure 1-2, Table 1). Shoot elongation was the parameter most significantly influenced by sugar concentration in the experiment. This, combined with observational recordings of health, were ultimately the best indicator of optimal sugar type and concentration.

For the cultivars tested, some carbon sources showed a general positive, or negative, effect, while others showed cultivar-specific responses. Erythritol was clearly detrimental to both cultivars, with survival rate and shoot proliferation extremely poor, eventuating in death. Although mature shoots on xylose and glycerol showed sufficient shoot elongation in some concentrations, morphogenic abnormalities were observed, which may partly be due to carbohydrate concentration affecting the osmotic potential of the medium (Cuenca & Vieitez 2000). It has been reported that high osmotic potential reduces plant height and growth (Cuenca & Vieitez 2000). However, this has not been confirmed for the concentration range considered in the present study, and warrants further study.

The excellent shoot growth for mature cv. Velvick on sucrose aligns with the universal role of sucrose in tissue culture. Sucrose is not only involved energy metabolism, but in regulating the expression of several enzymes and proteins that stimulate plant growth by inhibiting proteinases; hence growing and differentiating tissues are often found in the presence of high sucrose (Hassan, Gadalla & Abd-El Kareim 2008); (Swamy, M Kumara et al. 2010). Moreover, sucrose uptake efficiency across the plasma membrane is relatively high, and it hydrolysed into the metabolically active monosaccharides, glucose and fructose, by cell wall-bound invertases before being internalised (George *et al*., 2008). Unlike cv. Velvick, the relatively improved shoot growth of cv. Kidd on sorbitol highlights the problems associated with cultivar-specific optimisation of culture conditions in avocado. Inter-cultivar variations in sugar-use efficiency have been reported in other species, including apricot (Prunus armeniaca) and crabapple (Malus spp.) (Albrecht, 1986; Marino et al., 1993). Here, shoot production varied on sorbitol compared with sucrose in a variety-dependent manner, possibly. due to variation in specific enzymes for sorbitol oxidation (Ahmad, T. *et al*. 2007; Albrecht, 1986; Marino et al. 1993). Thus better shoot proliferation of cv. Kidd on sorbitol-containing medium may be associated with variation in the production of sorbitol hydrolysis enzymes.

The data here show a clear and variety-dependent role for sugars in avocado development and survival in culture. Future endeavours will assay the effects of supplementing C7 sugars; reported to be the major primary products of photosynthesis in avocado, in an attempt to further reduce inter-cultivar variations. However, while scientifically interesting, application of 7C sugars to a cost-effective tissue culture pipeline may be unlikely due to their prohibitive expense. Nonetheless, a better understanding of sugar-use efficiency in cultured avocado may improve the potential to develop an industry-standard protocol for avocado propagation via sterile culture.

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VIII CONGRESO MUNDIAL DE LA PALTA 2015

del 13 al 18 de Septiembre. Lima, Perú 2015

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