

## The Effect of MS Media Strength and Cytokinin in the Induction of Shoots from Shoot Tip Explants of Australian Finger Lime (*Citrus australasica* cv. Tasty Green) (Kesan Kekuatan Medium MS dan Sitokinin dalam Aruhan Pucuk daripada Hujung Pucuk Eksplan Limau Jejari Australia (*Citrus australasica* cv. Tasty Green))

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### ABSTRACT

*The Australian Finger Lime (Citrus australasica) is a type of citrus from the Rutaceae family, endemic to the east coast of Australia. The finger lime, loaded with numerous vitamins and renders a unique taste, has also been backed by science to contain essential amounts of antioxidants that are beneficial for cell protection, immune response, cancer prevention, ageing, arthritis and prevention of kidney stones. Current propagation attempts still rely on conventional methods that are less efficient and resulted in the slow establishment of farms for finger lime especially for commercialization purposes. This study focuses on the induction of shoots from shoot tip explants using 6-Benzylaminopurine (BAP) and Kinetin. Aseptic explants were inoculated into Murashige and Skoog (MS) medium of full-strength and half-strength followed by full-strength MS media supplemented with different concentrations of BAP and Kinetin. Results obtained in this study showed no significant differences in terms of the number of axillary shoots produced between explants cultured in full and half-strength MS media. However, the highest number of shoots and increment in shoot length were obtained from MS media supplemented with 2.0 mg/L BAP with the values  $1.80 \pm 0.27$  and  $2.56 \pm 0.36$  cm, respectively. In conclusion, MS media supplemented with 2.0 mg/L BAP was found optimal in the induction of shoots and shoot elongation of *C. australasica* cv. Tasty Green.*

*Keywords: 6-Benzylaminopurine; Australian Finger Lime; axillary shoots; Citrus australasica; kinetin*

### ABSTRAK

*Citrus australasica yang dikenali sebagai Limau Jejari Australia adalah daripada famili Rutaceae yang biasanya terdapat di pantai timur Australia. Limau ini kaya dengan pelbagai jenis vitamin dan mempunyai rasa yang unik, serta telah terbukti secara saintifik mempunyai kandungan antioksidan yang penting untuk perlindungan sel, tindak balas imun, perlindungan terhadap kanser, penuaan, arthritis dan pencegahan batu karang. Propagasi pokok limau ini masih bergantung pada kaedah konvensional yang kurang cekap dan menyukarkan penubuhan ladang limau ini terutamanya untuk tujuan komersial. Kajian semasa memfokuskan kepada penginduksian pucuk daripada eksplan pucuk menggunakan Benzilaminopurina (BAP) dan Kinetin. Eksplan steril dikultur dalam media Murashige dan Skoog (MS) pada kekuatan penuh dan separuh, serta MS berkekuatan penuh dengan penambahan BAP dan Kinetin. Keputusan daripada kajian ini menunjukkan tiada perbezaan yang signifikan dari segi jumlah pucuk aksilari yang dihasilkan antara eksplan yang dikultur dalam media MS kekuatan penuh dan separuh. Walau bagaimanapun, bilangan pucuk dan pertambahan ketinggian eksplan diperolehi dalam media MS pada kekuatan penuh ditambah dengan 2.0 mg/L BAP dengan nilai masing-masing  $1.80 \pm 0.27$  dan  $2.56 \pm 0.36$  cm. Kesimpulannya, media MS yang ditambah dengan 2.0 mg/L BAP didapati lebih optimum daripada rawatan yang lain dalam penginduksian pucuk dan pemanjangan pucuk *C. australasica* cv. Tasty Green.*

*Kata kunci: 6-benzilaminopurina; Citrus australasica; kinetin; Limau Jejari Australia; pucuk aksilari*

## INTRODUCTION

The Finger Lime (*Citrus australasica*), a fruit resembling finger-like shape, is placed amongst four other native citrus species, endemic to the rainforests in the east coast of Australia (Mabberley 1998). The other common name, caviar lime, is also attributed to this species as the globular vesicles of the pulp resemble caviar. Finger limes are grouped into the same family as other citruses, namely Rutaceae (Delort & Jaquier 2009).

Finger lime fruit is equipped with a unique shape, aroma, and colour (Delort & Jaquier 2009). This distinctive citrus species life form is generally shrub-like and can develop into a small tree up to 6 meters in height. Morphologically, the leaves are smaller compared to other citruses, and they have thorny stems. The small leaves and fruit peel are enriched with aromatic oil glands (Koskinen 2016). The fruit is cylindrical, and the flowers are typically white with small seeds produced that are whitish cream. This species harbours various cultivars that contribute to the diversity in pulp colours ranging from green, brown, pink and red (Koskinen 2016). Some of the established cultivars include the Alstonville, Blunobia Pink Crystal, Durham's Emerald, Judy's Everbearing, Pink Ice, Byron Sunrise and Jali Red, and these cultivars are currently registered under the Australian Cultivar Registration Authority (ACRA) (Delort et al. 2015).

Finger lime which contains juicy vesicles resembling caviar can be either directly consumed or used as a garnish as it provides a unique flavour element. Fresh fruits can be juiced or used as a flavouring agent in various food products (Delort et al. 2015). The finger lime fruits are expensive but the demand for these fruits has resulted in a boost of its export price for the domestic fresh fruit market (Hardy et al. 2010). The high nutritional content in this fruit gives an important role in various ailments. Kawaii et al. (2001) reported the presence of the Byangelicin compound in finger lime, is linked to the prevention of cell damage, encouraging cell regeneration and reducing the risk of cancer. The presence of phenolic compounds and significant antioxidants in the finger lime fruit was found to aid cell protection, as well as in reducing oxidative stress associated with diseases such as cancer, cardiovascular diseases, and neurodegenerative diseases (Netzel et al. 2007; Pham-Huy et al. 2008). Vitamin C is present abundantly in all cultivars and, the pink variety was found to be three times higher than that of in mandarin oranges (Konczak et al. 2010). Vitamin C is known to be highly capable of scavenging free radicals and provides photo-protection from UVA and UVB (Stamford 2012). Vitamin

E on the other hand is also present in finger limes and has been found to be linked to cancer prevention, ageing, arthritis, and cataract (Rizvi et al. 2014).

Finger lime plants are propagated through conventional methods similar to other citruses, such as grafting, seed germination or cuttings. Nevertheless, these methods in finger lime cultivation were more futile or less effective in producing healthy or viable cuttings. Other conventional methods such as budding and grafting rendered a high susceptibility to virus infection from the original mother plant (Shende & Manik 2015). Hardy et al. (2010) also highlighted the vulnerability of the finger lime species to various diseases and pests such as aphids, bronze orange bugs, caterpillars, snails, katydids, and grasshoppers resulting in reduced quality and quantity of fruit production. Besides, fungal infection such as melanose, gummosis, phytophthora, and dry fusarium rot significantly impacts plant health, thereby reducing fruit yield (Hardy et al. 2010). Issues involving this species that is prone to such pests and diseases severely impedes attempt for commercialization at a massive fruit production scale to cater global demand (Yasmina & Fazal 2016).

Plant tissue culture is an efficient alternative in mass propagating numerous elite plants yielding genetically identical clones of the mother plants. This technique varies in terms of culture conditions, plant growth regulators and plant media to optimise the production of desired propagules. *C. australasica* is commonly grown in the northeast of Australia and is not widely grown due to the low availability of plant stocks. Conventional methods applied in propagation finger lime plants such as grafting and cuttings are of low efficiency, tedious, and can be time consuming. Previous studies on tissue culture of citrus reported the suitability of MS media with the supplementation of different cytokinins such as BAP and Kinetin singly or in combination to induce multiple shoots particularly for micropropagation purposes (Fatonah et al. 2018; Silva et al. 2010). Therefore, the current study aims to evaluate the effects of MS media strength and the supplementation of cytokinin such as BAP and Kinetin in the induction of shoots from axillary shoot tip explants *C. australasica* cv. Tasty Green for future micropropagation attempts especially for the commercialization of this citrus.

## MATERIALS AND METHODS

## SOURCE OF EXPLANTS

The mother plants of *C. australasica* cv. Tasty Green

were obtained from the Superfruit Valley, Chuping, Perlis, Malaysia. The two-year-old plants were maintained at the School of Biological Sciences, Universiti Sains Malaysia under standard growing practices and fertilized weekly with NPK fertilizers at a ratio of 13:7:13. Young shoot tips of *C. australasica* cv. Tasty Green at the length of 3 cm long were excised and the thorns were removed prior to surface sterilization.

#### EXPLANT SURFACE STERILIZATION

Shoot tip explant were immersed in tap water containing 4% (v/v) Sunlight® dishwashing detergent liquid added with a few drops of tween-20 for 10-15 min with gentle brushing. The explants were then rinsed under running tap water for 30 min to remove detergent residues and soil particles. The cleaned shoot tip explants were sterilized by agitation in 70% (v/v) ethanol for 1 min, followed by 80% of commercial bleaching solution (Clorox® Regular-Bleach) for 7 min and rinsed 5 times with sterile distilled water. The sterile explants were blot dry on filter paper for 5 min and then excised into approximately 2 cm length before inoculated on full-strength MS (Murashige and Skoog) media. All explants were maintained in culture at 16 h light and 8 h dark photoperiod and  $25 \pm 2$  °C under white fluorescent light (Philips TLD, 36 W.  $150 \mu\text{mol m}^{-2}\text{s}^{-1}$ ).

#### THE INDUCTION OF SHOOTS IN DIFFERENT STRENGTHS OF MS MEDIA

Sterile shoot tip explants were cultured on both full and half-strength MS media without plant growth regulators. The number of shoots induced from both media were recorded and analysed after 6 weeks of culture.

#### SHOOT INDUCTION USING BAP AND KINETIN

The sterile shoot tip explants were inoculated on full-strength MS media supplemented with different concentrations of BAP (0.1, 0.5, 1.0, 2.0, 3.0, and 4.0 mg/L) and Kinetin (0.1, 0.5, 1.0, 2.0, 3.0, and 4.0 mg/L). The explants were sub-cultured after 4 weeks. The percentage of shoot induction, the number of shoots induced per explant and the average length of induced shoots were evaluated after 6 weeks of culture.

#### STATISTICAL ANALYSIS

All data were analysed using IBM SPSS Statistical 24 software. Data obtained from the induction of shoots in different strengths of MS media was subjected to

Independent T-test, whereas the data obtained from the induction of shoots using BAP and Kinetin were subjected to One-Way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test at the significance level of  $p \leq 0.05$ .

## RESULTS AND DISCUSSION

#### THE INDUCTION OF SHOOTS IN DIFFERENT STRENGTHS OF MS MEDIA

Sterile shoot tips of *C. australasica* were cultured on full-strength and half-strength MS media. The percentage of shoots induced from shoot tip explants on full and half-strength media were 65 and 60%, respectively, after 6 weeks of culture. With reference to Table 1, there is no significant difference between these values, indicating that both MS media strength can be used throughout the study.

Previous works of literature on citrus has reported the preference of using full-strength MS media for shoot induction and half-strength MS media for root induction. A recent study conducted on *C. australasica* used full-strength MS media for the induction of shoots, whereas half-strength MS media was used to induce roots (Mahmoud et al. 2020). The utilisation of full-strength MS media in rendering the maximum number of shoots induced were also observed in various *Citrus* species, such as *Citrus aurantifolia* with the average of 8 shoots per node (Al-Khayri & Al-Bahrany 2001) and *Citrus megaloxycarpa* with the mean number shoots of 4.7 (Haripyaree et al. 2011). Other citrus plants were also found to produce a maximum number of *in vitro* shoots in full-strength MS media such as *Citrus reticulata* at 3.4 shoots (Shende & Manik 2015), *Citrus halimii* at 3.7 shoots (Normah et al. 1997) and *Citrus limon* L. cv. Kaghzi Kalan at 5.5 shoots per explant (Goswami et al. 2013). The reason behind full strength media rendering only 1.35 shoots can be attributed to the fact that other citrus species as reported by Vibhute et al. (2012) genotypically responded to the full-strength media, thereby resulting in differences to the number of induced shoots.

With reference to the work of Rathore et al. (2007), MS media with half the concentration of ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ) and potassium nitrate ( $\text{KNO}_3$ ) rendered optimal performance at  $18 \pm 2.12$  shoots when the nodal shoot segments of *Citrus lemon* were cultured, whereas half-strength MS media was used for the root induction of *Citrus jambhiri* Lush.

TABLE 1. The percentage of shoot induction and average number of induced shoots from shoot tips of *Citrus australasica* cv. Tasty Green using full-strength and half-strength MS media

Strength of media	Percentage of shoot induction (%)	Average number of induced shoots (N) ( $\bar{x} \pm s.e$ )
Full-strength	65	1.35 $\pm$ 0.27 <sup>a</sup>
Half-strength	60	0.85 $\pm$ 0.20 <sup>a</sup>

\*Column with same letter indicates that there was no significant difference between mean. (Independent T test,  $p \leq 0.05$ )

#### THE INDUCTION OF SHOOTS WITH PLANT GROWTH REGULATOR BAP AND KINETIN

Full-strength MS media was used to induce shoots with plant growth regulators BAP and Kinetin, in agreement with previous studies on citrus reporting on the superiority of full-strength over half-strength MS media in producing a higher number of regenerated shoots for citrus plants (Eed et al. 2011; Taye et al. 2018). Thus, full-strength MS media was selected for the induction of shoots in this investigation.

In the current study, the percentage of induced shoots for all treatments was at the range of 55 to 80%, thereby indicating a high suitability of the full-strength MS media supplemented with plant growth regulators in facilitating explant growth. MS media supplemented with 2.0 mg/L of BAP particularly rendered the highest percentage of shoot induction, at 80%. With reference to Table 2, shoot tips explants supplemented with 2.0 mg/L BAP produced the highest number of shoots and the highest average height of induced shoots at  $1.80 \pm 0.27$  and  $2.56 \pm 0.36$  cm, respectively. BAP at 2.0 mg/L was found to be the optimal concentration to induce shoots, increment of shoot length, as compared to other treatments in this study.

The optimal number of shoots at 1.80 is considered low when compared to other citrus species. However, the *in vitro* production of shoots may reflect upon the slow growth nature of the finger lime trees in the wild (Hardy et al. 2010). Figure 1 depicts the axillary shoots formed from shoot tip explants on different treatments of BAP after 6 weeks of culture. The number of shoots decreased when the concentration of BAP was raised to 3.0 and 4.0 mg/L. This could be due to the fact that a higher concentration of BAP would interfere and disrupt optimal induction and proliferation of shoots *in vitro* (Nelson et al. 2015). With reference to Martin et al. (2006) and Venkatachalam et al. (2007), high concentrations of BAP can induce potential

somaclonal variation resulting in abnormal phenotypes and non-true-to-type plants.

Mahmoud et al. (2020) reported that MS media supplemented with 2.2  $\mu$ M of BAP produced the highest percentage of shoot induction (above 80%) and the treatment of MS media supplemented with 2.2  $\mu$ M of BAP, 60  $\mu$ M of silver thiosulfate (STS) and 0.70  $\mu$ M of gibberellic acid ( $GA_3$ ) resulted in the highest number of shoot induction with an average of four shoots per explant. Previous studies on other *in vitro* citrus also highlighted the efficiency of BAP in inducing multiple shoots as compared to other cytokinins. Haripyaare et al. (2011) reported that the highest number of multiple shoot formation was observed in the combination of 0.25 mg/L BAP and 0.50 mg/L NAA ( $4.7 \pm 0.5$  shoots per explant), followed by 1.0 mg/L BAP and 0.50 mg/L Kinetin ( $4.4 \pm 0.4$  shoots per explant) from shoots tip explants of *C. megaloxycarpa*. Shoot tip explants of pummelo (*Citrus grandis* L. Osbeck) was reported to produce an average of 5.2 shoots when treated with 1.8  $\mu$ M of BAP (Paudyal & Haq 2000). Meanwhile, Singh et al. (1994) discovered that MS media supplemented with 1.0 mg/L BAP, 0.5 mg/L Kinetin and 0.5 mg/L NAA gave the highest multiple shoot number and shoot length ( $6.1 \pm 0.11$  and  $2.6 \pm 0.09$  cm, respectively) from shoot tips of *C. reticulata*. This treatment also worked optimally for *C. limon* explants in terms of the number of shoot multiplication, shoot length and number of leaves (Singh et al. 1994). Another study from Bhagat et al. (2012) reported that 3.0 mg/L BAP is optimal in inducing multiple shoots (4.02 shoots) and increasing shoot height (1.81 cm) for *C. jambhiri*. Besides, both shoot tips and nodal segments of sweet orange cv. Mosambi produced the maximum shoot length and highest number of leaves from combination of 2.0 mg/L BAP, 1.0 mg/L Kinetin and 0.1 mg/L NAA (Upadhyay et al. 2010). With reference to the reports as above, it was evident that BAP plays an important role in the induction of shoots and shoot elongation for various citrus cultivars.

On the other hand, certain citrus species was reported to respond to Kinetin in terms of the initiation of shoots. Goswami et al. (2013) reported a lower number of shoots per explant at  $1.2 \pm 0.14$  shoots in the treatment of MS media supplemented with 0.5 mg/L of Kinetin. Similar result was obtained in a study conducted by De Oliveira et al. (2016), whereby MS media supplemented with 1.0 mg/L of Kinetin rendered a mean number of 1.12 shoot for *Citrus sinensis* cv. Washington Navel. However, Mukhtar et al. (2005) reported that shoot tips of *Citrus reticulata* (Blanco) rendered the highest number of shoots at 7.99 shoots in the treatment of MS media supplemented with 1.5 mg/L Kinetin. In the current study, 3.0 mg/L of Kinetin rendered  $1.25 \pm 0.23$  shoots, which is in contrast with the high induced shoot number as reported by Mukhtar et al. (2005).

The vast differences in the number of shoots produced can be attributed to the fact that the presence of endogenous hormones in plant cells influences the *in vitro* organogenesis of shoots. Hu et al. (2017) reported that the inhibition of *in vitro* shoot organogenesis in the basal end of citrus epicotyl was due to the presence of elevated levels of endogenous auxin. Besides, the synergistic and inhibitory interactions of exogenous and endogenous plant growth regulators are specific, as different species, genotype and explant source largely influences the response of plant cells and tissues (Mwaniki et al. 2019). Nevertheless, this signifies that both cytokinins, BAP, and Kinetin are commonly used to induce multiple shoots for most citrus species, with preference given to BAP in most literatures due to its superiority over Kinetin in inducing a higher number of shoots.

TABLE 2. The percentage of shoot induction, average number of induced shoots and average height of induced shoots on media supplemented with BAP and Kinetin

Treatment (mg/L)	Percentage of shoot induction (%)	Average number of induced shoots (N) ( $\bar{x} \pm S.E$ )	Average height of induced shoots (cm) ( $\bar{x} \pm S.E$ )
0.0	65	$1.00 \pm 0.22^b$	$0.99 \pm 0.21^b$
0.1 BAP	65	$1.10 \pm 0.22^b$	$1.16 \pm 0.22^b$
0.5 BAP	65	$1.00 \pm 0.22^b$	$1.20 \pm 0.27^b$
1.0 BAP	60	$1.00 \pm 0.22^b$	$1.09 \pm 0.25^b$
2.0 BAP	80	$1.80 \pm 0.27^a$	$2.56 \pm 0.36^a$
3.0 BAP	70	$1.10 \pm 0.22^b$	$1.19 \pm 0.21^b$
4.0 BAP	60	$0.80 \pm 0.19^b$	$1.13 \pm 0.26^b$
0.1 Kinetin	60	$0.80 \pm 0.17^b$	$0.91 \pm 0.21^b$
0.5 Kinetin	60	$0.90 \pm 0.22^b$	$0.87 \pm 0.21^b$
1.0 Kinetin	65	$1.00 \pm 0.21^b$	$0.97 \pm 0.19^b$
2.0 Kinetin	55	$0.70 \pm 0.16^b$	$0.95 \pm 0.20^b$
3.0 Kinetin	70	$1.25 \pm 0.23^{ab}$	$1.18 \pm 0.22^{ab}$
4.0 Kinetin	60	$1.15 \pm 0.24^b$	$1.18 \pm 0.24^b$

\*Column with same letter indicates that there was no significant difference between means. (Duncan's test,  $p \leq 0.05$ )



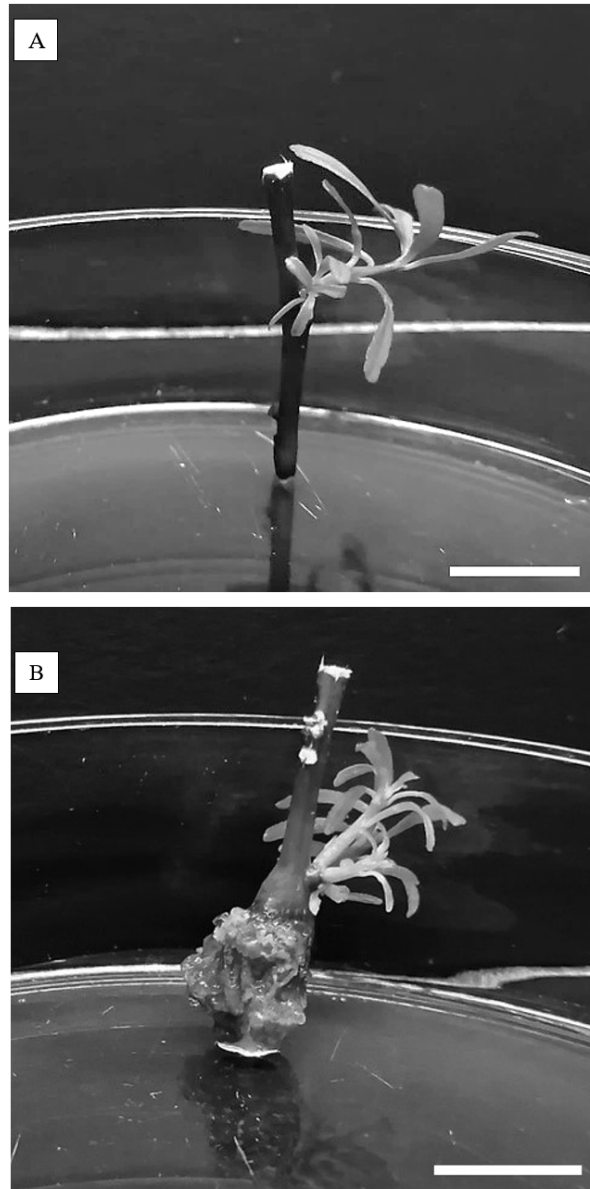


FIGURE 1. The induction of shoots from shoot tip explants in different treatments of BAP after 6 weeks of culture (A) Control, MS media (B) MS media supplemented with 2.0 mg/L BAP. Scale bars represent 1 cm

#### CONCLUSION

Sterile cultures and the induction of shoots from shoot tip explants of *C. australasica* cv. Tasty Green were successfully established in this study. The current report also indicated that there was no significant difference in

terms of the number of induced shoots for full and half-strength MS media. The treatment of full-strength MS media supplemented with 2.0 mg/L BAP resulted in the highest number of induced shoots and shoot elongation as compared to other BAP and Kinetin treatments.

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