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SHOOT REGENERATION OF LIMAU PURUT (*CITRUS HYSTRIX*) USING SHOOT TIP: ASSESSMENT OF CALCIUM GLUCONATE AND SILVER NITRATE IN OVERCOMING PREMATURE LEAF SENESCENCE

WEE HIANG ENG[1], MAHERAN ABD AZIZ[1,2*] AND UMA RANI SINNIAH[3]

[1]*Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia*
[2]*Laboratory of Plantation Crops, Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia*
[3]*Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia*
[*]*Corresponding author e-mail: maheran@agri.upm.edu.my*

Abstract

This study was carried out to establish an optimum *In vitro* shoot multiplication system using shoot tip explants derived from 7 week-old seedlings of *Citrus hystrix*. In the first experiment, shoot tips were cultured on Murashige & Skoog (MS) medium supplemented with 0-13.33 µM 6-benzylaminopurine (BAP) for 8 weeks. Shoot tips cultured on 2.22 µM BAP produced the highest mean number of shoots (3.42 shoots) but the shoots had low number of leaves (1.14 leaves) due to the occurrence of premature leaf senescence and callus formation. Meanwhile, the medium devoid of BAP produced the lowest mean number of shoots (1.50 shoots) but highest mean number of leaves (5.41 leaves) indicating that BAP was likely responsible for the premature leaf senescence. In order to overcome the occurrence of premature leaf senescence on medium with BAP, a second experiment was carried out whereby shoot tips were cultured on medium containing 2.22µM BAP fortified with 2.00, 4.00 and 6.00 mM calcium gluconate (Ca-glu) and a control treatment with 2.22 µM BAP. The shoot and leaf numbers were increased with the addition of 4.00 and 6.00 mM Ca-glu. The presence of Ca-glu reduced premature leaf senescence and callus formation to some extent. In the third experiment, the addition of silver nitrate ($AgNO_3$) at 10–80 µM in media with 2.22 µM BAP and 2.22 µM BAP + 4 mM Ca-glu could totally overcome premature leaf senescence and callus formation. Media supplemented with 2.22 µM BAP + 4 mM Ca-glu + 20 µM $AgNO_3$ significantly induced among the highest mean number of shoots and highest mean number of leaves per shoot.

Introduction

Citrus hystrix belongs to the family Rutaceae and genus Papeda (Nicolosi, 2007). There is vast potential for commercialization of this underutilised species for its nutritional, medicinal and antimicrobial properties. According to Ghafar *et al*., (2010), *C. hystrix* has the highest amount of antioxidant compared to other commonly consumed citrus in Malaysia like *C. aurantifolia, C. sinensis* and *C. microcarpa*. For citriculture farmers, *C. hystrix* can be used as rootstock or interstock to *C. reticulata* to increase resistance against greening disease (Shokrollah *et al*., 2010). These noble characters are very important to the citrus industry as they offer vast potential for citrus improvement via conventional breeding and biotechnology.

Shoot tip and node can be stimulated to branch and produce axillary shoots when cultured on medium supplemented with the appropriate concentration of cytokinin (George & Debergh, 2008). Propagation based on axillary shoot proliferation tends to reduce the occurrence of somaclonal variation compared to adventitious shoot regeneration from callus (Preece, 1997). The economic loss due to somaclonal variation in citrus is high since abnormalities could only be noticed after many years of cultivation in the field (Rani & Raina, 2000). Most researches in citrus micropropagation focus on finding the optimum composition of medium to control shoot and root organogenesis, callus cultures and somatic embryogenesis. The optimum concentration of cytokinin and auxin for shoot multiplication varies considerably among citrus species but much similar between cultivars that belong to the same species (Marin & Duran-Vila, 1991).

This paper reports on multiple shoot induction of *C. hystrix* and the application of Ca-glu and $AgNO_3$ to overcome the occurrence of premature leaf senescence. This valuable yet underutilised species demands urgent investigation on its micropropagation in order to reap its full potential. An efficient micropropagation system not only provides a mean for mass propagation but also a tool in genetic modification.

Material and Methods

Surface sterilization and plant material preparation: Mature fruits of *C. hystrix* were harvested from plants maintained at the Agrobiotechnology Laboratory, Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Malaysia. Seeds were extracted from the fruits and surface sterilized using 20% (v/v) Clorox with a few drops of Tween-20 for 20 min and rinsed three times in sterile distilled water. The seeds were inoculated in vials (10.0 cm long and 2.5 cm diameter) containing 10mL of MS (Murashige & Skoog, 1962) medium. Shoot tips with two node, measuring 0.8-1.0 cm in length, were excised transversely from 7 week-old *in vitro* germinated seedlings for use as explants.

Shoot multiplication medium and culture condition: In all the experiments, MS basal salt was employed in combination with different concentrations of 6-benzylaminopurine (BAP), calcium gluconate (Ca-glu) and/or silver nitrate ($AgNO_3$). In the first experiment, MS medium was supplemented with 0.55, 1.11, 2.22, 4.44, 6.66, 8.88, 11.10 and 13.32 µM BAP and the control

treatment was MS medium without BAP. In the second experiment, MS medium was supplemented with 2.22 µM BAP in combination with 2.00, 4.00 and 6.00 mM Ca-glu and the control treatment was MS + 2.22 µM BAP. In the third experiment, 10.00, 20.00, 40.00 and 80 µM $AgNO_3$ were supplemented to MS + 2.22 µM BAP and MS + 2.22 µM BAP + 4.00 mM Ca-glu, with MS + 2.22 µM BAP as the control. All media used in the experiments were supplemented with 3% (w/v) sucrose, 0.28% (w/v) phytagel and the pH was adjusted to 5.7 with 1N NaOH prior to autoclaving at 121°C for 20 min. $AgNO_3$ was filter sterilized and added to the autoclaved media.

Shoot tips were cultured upright in 250 mL jam jar containing 50 mL of medium. The experiments were repeated 4 times and each treatment per replicate contained 6 explants. In all experiments, shoot tips were subcultured at 4-week intervals. Cultures were incubated at 25 ± 1°C with 16 hours photoperiod (40 µmol $m^{-2}s^{-1}$) provided by cool white fluorescent tubes.

Statistical analysis: The experiments were set up in Randomized Complete Block Design (RCBD). Data were subjected to normality test before analysis of variance (ANOVA) using SAS 9.1 programme. For those variables that were not normal, data transformation was applied. When treatments were significantly different, means were separated at the 5% level using Duncan's Multiple Range Test (DMRT).

Results and Discussion

Effects of various concentrations of BAP on shoot regeneration from shoot tip of *C. hystrix*: In this study, shoot tips responded with the emergence of axillary buds from the nodes after 2 weeks of culture on medium supplemented with BAP. Most of the leaves on the shoot exhibited senescence after 3 weeks of culture and some shoots produced callus before senescence. The leave-less shoots continued to produce lateral shoots (Fig. 1a) but shoots that produced callus (Fig. 1b) or underwent complete senescence died completely.

After 8 weeks of culture, shoot tips cultured on medium supplemented with 2.22µM BAP produced significantly the highest mean number of shoots (3.42 shoots), however, the mean number of leaves per shoot was low (1.14 leaves) (Table 1). The shoots were poor in quality and failed to grow and root in subsequent subculture (Fig. 1c). In contrast, medium devoid of BAP produced the lowest mean number of shoots per explant (1.5 shoots) and the lowest percentage of explants producing shoots (79.17%) but had significantly the highest mean number of leaves (5.41 leaves) and highest mean shoot length (1.21 cm) (Fig. 1d).

Medium supplemented with high concentration of BAP (13.32 µM) showed an inhibitory effect in terms of shoot number, percentage of explants producing shoots and shoot length. An increase in BAP concentration from 4.44 to 13.32 µM BAP not only caused a reduction in shoot proliferation but also lower mean number of leaves (Table 1). This result is in agreement with the finding on micropropagation of *Ochradenus arabicus*, where supra optimal level of cytokinin could reduce multiplication.

Although micropropagation of citrus has been carried out for several decades there is still room for improvement and optimization due to its vast genotype diversity. Micropropagation using juvenile tissues is a common practice due to relatively more responsive to a given treatment compared to mature explants (Memon *et al*., 2013) and ease of obtaining contamination free culture in *C. hystrix*. BAP is the most commonly used cytokinin in tissue culture for the genus citrus, but the optimum concentration for maximum proliferation varies among species. For instance, shoot tips of *C. mitis* requires 4.44 µM BAP (Sim *et al*., 1989), *C. grandis* requires 1.8 µM BAP (Paudyal & Haq, 2000) and *C. depressa*, *C. jambhiri* and *C. reshni* require 4.44 µM BAP (Sharma *et al*., 2009) for maximum shoot proliferation. Most researchers who reported the use of BAP alone or in combination with auxin were able to produce shoots that can successfully undergo rooting and acclimatization. Axillary shoot multiplication of *Scrophularia takesimensis* can be promoted using cytokinin alone, but high frequency multiplication can be achieved if auxin is combine with cytokinin. However, there is no report so far on the occurrence of leaf senescence during micropropagation of citrus derived from juvenile explants.

In this experiment, medium incorporated with BAP alone could stimulate shoot proliferation but inadequate to produce normal shoots that can reach the rooting stage. Most of the shoots generated tend to produce callus after the first subculture. Later, new lateral shoots emerged and elongated but producing abnormal leaves that eventually fell. The leaf lamina failed to expand with only the midrib weakly attached to the stem. The higher concentrations of BAP were found to hasten leaf senescence.

Effects of various concentrations of Ca-glu on shoot regeneration from shoot tip of *C. hystrix*: In order to overcome premature leaf senescence and to improve shoot multiplication of *C. hystrix*, various concentrations of Ca-glu were added into MS medium supplemented with 2.22 µM BAP (optimized in previous experiment). Medium supplemented with 4mM Ca-glu was able to significantly increase the mean number of shoots produced (3.67 shoots) compared to media supplemented with 2 mM Ca-glu and without Ca-glu (Table 2). Meanwhile, medium supplemented with 6mM Ca-glu did not significantly improve shoot production compared to medium supplemented with 4mM Ca-glu. The addition of Ca-glu had no significant effect on the percentage of explants producing shoot compared to the control. The study revealed that medium supplemented with Ca-glu could not completely prevent premature leaf senescence. Newly formed leaves remained weakly attached on the shoots and senesced later after subculture.

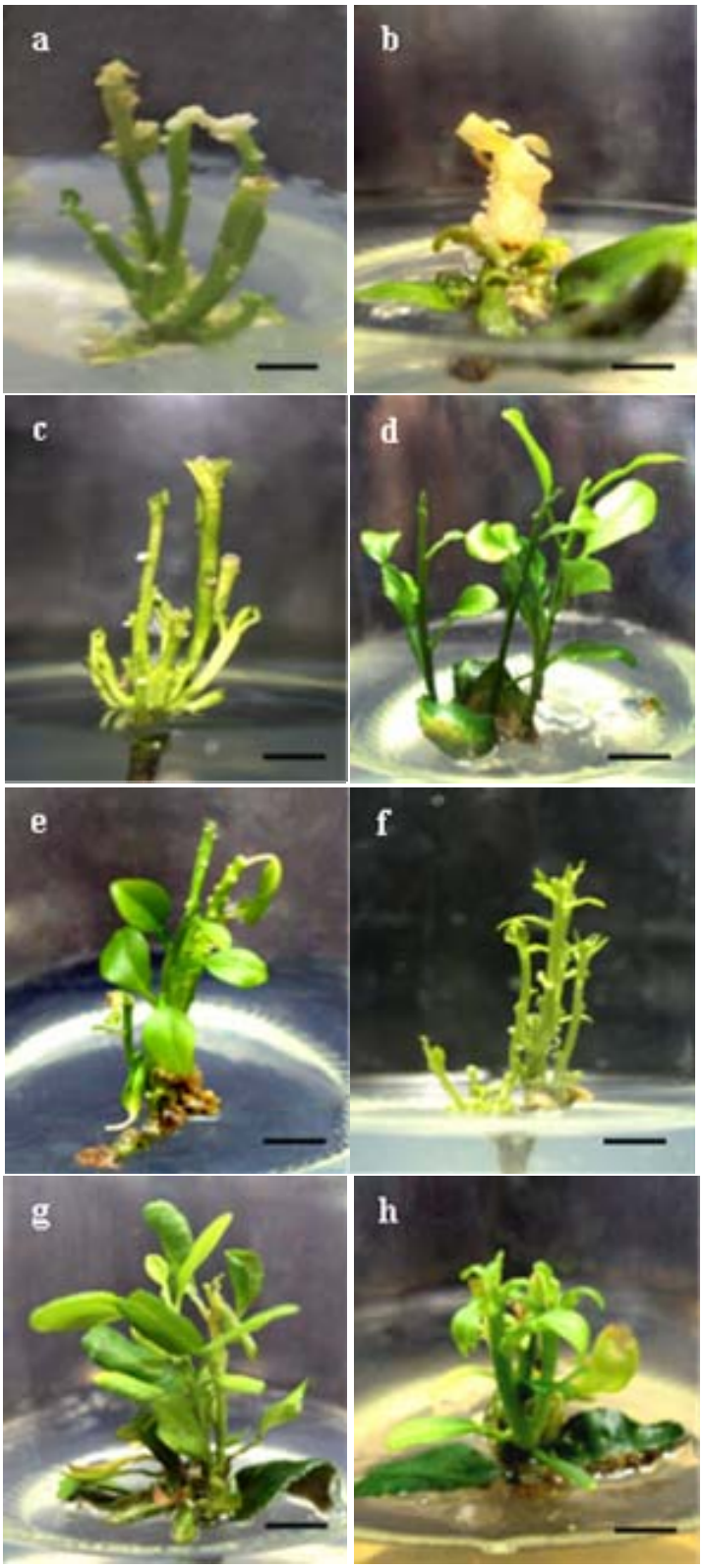

Fig. 1. Multiple shoot induction from shoot tip of *C. hystrix* after 8 weeks of culture (a) leafless shoot tip continued to produce lateral shoots, (b) callus formed on shoot surface, (c) poor shoot growth on 2.22 µM BAP, (d) healthy shoots on medium devoid of BAP, (e) larger leaves produced on 2.22 µM BAP + 4 mM Ca-glu, (f) smaller leaves produced on 2.22 µM BAP + 2 mM Ca-glu, (g) larger leaves produced on 2.22 µM BAP + 4 mM Ca-glu + 20 µM $AgNO_3$ and (h) smaller leaves produced on 2.22 µM BAP + 20 µM $AgNO_3$ (Bar = 5 mm).

Table 1. Effects of various concentrations of BAP on shoot regeneration from shoot of *C. hystrix* after 8 weeks of culture.

BAP (µM)	Number of shoots ± S.E	Number of leaves ± S.E ‡	Shoot length (cm) ± S.E	Percentage of explants producing shoot ± S.E †
0.00	1.50 ± 0.07^{e}	5.41 ± 0.18^{a}	1.21 ± 0.06^{a}	79.17 ± 4.17^{c}
0.55	1.96 ± 0.08^{cd}	2.37 ± 0.10^{b}	0.85 ± 0.06^{b}	100 ± 0.00^{a}
1.11	2.33 ± 0.07^{b}	1.28 ± 0.10^{c}	0.86 ± 0.03^{b}	100 ± 0.00^{a}
2.22	3.42 ± 0.05^{a}	1.14 ± 0.06^{c}	0.80 ± 0.03^{bc}	95.83 ± 4.17^{ab}
4.44	2.29 ± 0.08^{b}	0.85 ± 0.03^{d}	0.73 ± 0.05^{cd}	87.50 ± 4.17^{bc}
6.66	2.17 ± 0.07^{bc}	0.79 ± 0.04^{d}	0.79 ± 0.03^{bc}	91.67 ± 8.33^{ab}
8.88	2.00 ± 0.07^{cd}	0.63 ± 0.02^{e}	0.77 ± 0.05^{bc}	100 ± 0.00^{a}
11.10	1.96 ± 0.13^{cd}	0.53 ± 0.05^{ef}	0.68 ± 0.04^{cd}	100 ± 0.00^{a}
13.32	1.79 ± 0.08^{d}	0.42 ± 0.04^{f}	0.65 ± 0.04^{d}	87.50 ± 4.17^{bc}

‡ Data were transformed using (Log_{10} Y+1) prior to ANOVA and converted back to original scale for demonstration in the table (Comptom, 1994)

† Data were transformed using arc sine prior to ANOVA and converted back to original scale for demonstration in the table (Comptom, 1994)

Data are means of 4 replicates with 6 explants per replicate. Means followed by different alphabet denote significant differences within column based on DMRT (p = 0.05)

Table 2. Effects of various concentrations of Ca-glu on shoot regeneration from shoot of *C. hystrix* after 8 weeks of culture.

Ca-glu (mM)	Number of shoots ± S.E	Number of leaves ± S.E	Shoot length (cm) ± S.E	Percentage of explants producing shoot ± S.E †
0.00	3.21 ± 0.12^{b}	1.05 ± 0.07^{c}	0.81 ± 0.04^{ab}	100 ± 0.00^{a}
2.00	3.17 ± 0.07^{b}	1.73 ± 0.12^{b}	0.84 ± 0.03^{a}	91.67 ± 4.81^{a}
4.00	3.67 ± 0.18^{a}	2.10 ± 0.02^{a}	0.74 ± 0.02^{b}	100 ± 0.00^{a}
6.00	3.42 ± 0.05^{ab}	1.65 ± 0.07^{b}	0.61 ± 0.03^{c}	95.83 ± 4.17^{a}

All media contained 2.22 µM BAP.

† Data were transformed using arc sine prior to ANOVA and converted back to original scale for demonstration in the table (Comptom, 1994).

Data are means of 4 replicates with 6 explants per replicate. Means followed by different alphabet denote significant differences within column based on DMRT (p = 0.05).

It was found that the supplementation of Ca-glu in medium containing 2.22 µM BAP could improve the shoot quality of *C. hystrix* by producing higher number of leaves and reducing premature shoot senescence to some extent. The larger sized leaves formed on medium with 2.22 µM BAP + 4 mM Ca-glu (Fig. 1e) in comparison to those formed on medium supplemented with 2.22 µM BAP + 2 mM Ca-glu (Fig. 1f) indicated improved leaf expansion at higher concentration of calcium. Beemster *et al*., (2003) stated that cell multiplication and cell expansion will lead to leaf blade expansion. Ca^{2+} strengthens the cell walls that will increase the cell tolerance against biotic and abiotic stress. Ca^{2+} also stabilizes cell membrane by connecting various protein and lipids at the membrane surface. Physiologically, Ca^{2+} is involved in cell elongation, cell division and influences the pH of the cells and as a regulatory ion in the source sink translocation carbohydrate (Hawkesford *et al*., 2012). Russell and McCown (1988) supplemented 6 mM Ca-glu to woody plant medium (WPM) (Lloyd & McCown, 1981) to rectify Ca^{2+} deficiency, without altering the concentrations of the customary anions in WPM medium for *Populus* spp. culture.

Effects of various concentrations of $AgNO_3$ with/without 4 mM Ca-glu on shoot regeneration from shoot tip of *C. hystrix*: No premature leaves senescence and callus formation were observed in all media supplemented with 10 to 80 µM of $AgNO_3$. Treatments supplemented with 20 µM $AgNO_3$ with/without 4 mM Ca-glu produced among the highest mean number of shoots (4 shoots) (Table 3). Treatments containing 2.22 µM BAP + 4 mM Ca-glu + 20 µM $AgNO_3$ and 2.22 µM BAP + 40 µM $AgNO_3$ produced among the highest mean number of leaves per shoot (4 leaves). Generally, the use of low level of $AgNO_3$ (10 µM) was adequate to prevent premature leaf senescence in *C. hystrix*. However, the addition of 20 µM $AgNO_3$ was recommended in order to achieve maximum shoot proliferation and simultaneously to overcome the premature leaf senescence. Larger leaves (Figs. 1g and 1h) and significantly higher mean number of leaves were obtained on medium with 2.22 µM BAP + 4 mM Ca-glu + 20 µM $AgNO_3$ as compared to medium with 2.22 µM BAP + 20 µM $AgNO_3$ (Table 3). The high concentration of 80 µM $AgNO_3$ particularly with the inclusion of 4 mM Ca-glu showed inhibitory effect on shoot regeneration from the shoot tips. Shoots produced were of poor quality and low in number, producing few leaves that appeared abnormal and glassy.

According to Woeste *et al*., (1999) and Vandenbussche *et al*., (2003), the production of ethylene can be stimulated by the application of exogenous synthetic hormones like auxins, cytokinins, gibberellins and abscisic acid. The synergistic effect between synthetic hormones and ethylene causes premature leaf senescence. In addition, explants release ethylene due to wounding during explanting, subculture and the composition of medium also can give rise to ethylene production (Reid, 1995). In this study, the occurrence of premature leaf senescence was effectively overcome by the addition of $AgNO_3$. The detrimental effect of ethylene probably induced by BAP during the shoot multiplication stage of *C. hystrix* was removed by $AgNO_3$. Silver cation from $Ag_2S_2O_3$ and $AgNO_3$ are inhibitors of ethylene action on the whole plant (Moshkov *et al*., 2008). This result explained that inclusion of BAP during the shoot multiplication stage had stimulated the production of ethylene that caused substantial premature leaf senescence.

The vessels used in this study were capped with transparent polypropylene cap and sealed with parafilm that limited ventilation. As a result, ethylene may accumulate within the headspace of the vessel. Tholen *et al*., (2004) showed that in well ventilated conditions ethylene-insensitive genotypes of *Arabidopsis*, tobacco and petunia had no larger total leaf area compared to ethylene-sensitive control plants. This shows that the accumulation of ethylene in the headspace of tissue culture vessels will inhibit leaf expansion on ethylene-sensitive plants. In conclusion, 2.22 µM BAP + 4 mM Ca-glu + 20 µM $AgNO_3$ is the most suitable medium in this experiment in order to achieve optimum regeneration of healthy, normal and vigorous shoots.

In the subsequent subculture, individual shoots (0.8–1.0 cm length) derived from the shoot regeneration medium consisting of 2.22 µM BAP + 4 mM Ca-glu + 20 µM $AgNO_3$ were transferred into medium without hormone and medium with 2.22 µM BAP. After 8 weeks of culture, no premature leaf senescence was observed on the hormone-free medium indirectly indicating that BAP in the medium was the cause of premature leaf senescence. However, shoots cultured on 2.22 µM BAP showed leaf senescence after 6 weeks culture. The delayed occurrence of premature leaf senescence on medium with 2.22 µM BAP could be due to the carry over effects of $AgNO_3$ from previous culture medium.

Table 3. Effects of various concentrations of $AgNO_3$ with/without 4 mM Ca-glu on shoot regeneration from shoot of *C. hystrix* after 8 weeks of culture.

Treatment	Number of shoots ± S.E	Number of leaves ± S.E	Shoot length (cm) ± S.E	Percentage of explants producing shoot ± S.E
Control	3.38 ± 0.08^{b}	1.23 ± 0.07^{f}	0.78 ± 0.03^{a}	100 ± 0.00^{a}
10.00 µM $AgNO_3$	3.75 ± 0.11^{ab}	3.36 ± 0.05^{d}	0.56 ± 0.03^{b}	95.83 ± 4.17^{ab}
20.00 µM $AgNO_3$	4.00 ± 0.07^{a}	3.66 ± 0.05^{bc}	0.48 ± 0.03^{c}	100 ± 0.00^{a}
40.00 µM $AgNO_3$	2.88 ± 0.12^{c}	3.77 ± 0.04^{ab}	0.53 ± 0.02^{bc}	91.67 ± 4.81^{abc}
80.00 µM $AgNO_3$	2.63 ± 0.08^{c}	2.99 ± 0.06^{e}	0.50 ± 0.02^{bc}	87.50 ± 4.17^{bc}
4.00 mM Ca-glu + 10.00 µM $AgNO_3$	3.75 ± 0.16^{ab}	3.60 ± 0.03^{c}	0.46 ± 0.02^{c}	100 ± 0.00^{a}
4.00 mM Ca-glu + 20.00 µM $AgNO_3$	3.96 ± 0.08^{a}	3.91 ± 0.03^{a}	0.50 ± 0.04^{bc}	100 ± 0.00^{a}
4.00 mM Ca-glu + 40.00 µM $AgNO_3$	2.59 ± 0.05^{c}	3.63 ± 0.05^{bc}	0.46 ± 0.02^{c}	91.67 ± 4.81^{abc}
4.00 mM Ca-glu + 80.00 µM $AgNO_3$	1.75 ± 0.28^{d}	2.91 ± 0.03^{e}	0.46 ± 0.02^{c}	83.33 ± 0.00^{c}

All media contained 2.22 µM BAP includes control

Data are means of 4 replicates with 6 explants per replicate. Means followed by different alphabet denote significant differences within column based on DMRT (p = 0.05)

Conclusion

In conclusion, 2.22 µM BAP + 4 mM Ca-glu + 20 µM $AgNO_3$ was found to be the most suitable medium to achieve optimum proliferation of healthy, normal and vigorous shoots from juvenile shoot tips of *C. hystrix*. Further investigation in terms of genetic fidelity and pathological test need to be carried out before the system can be applied for mass propagation, germplasm conservation and genetic modifications of this species.

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