RESEARCH ARTICLE

Improved protocol for efficient regeneration of coconut (*Cocos nucifera* L.) anther derived embryos

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Abstract: The occurrence of severe shoot necrosis and other constraints such as low frequency of embryo induction and poor regeneration into plants, restrict the use of coconut androgenesis in practice. Fine-tuning of the protocol by addressing the above constraints was carried out with the intention of scaling-up haploid plant production. Out of the carbon types, sucrose and maltose, when added in concentrations of 90.0 gL^{-1} and 120.0 gL^{-1} showed significantly higher (p < 0.05) embryo (44.0 % and 36.0 %, respectively) production. Out of the concentrations used in the study, 20.0 µM 6-benzylaminopurine (BAP) showed significantly (p = 0.001) higher shoot generation (47.6 %) as well as significantly (p = 0.006) longer shoot production (31.7 %) during the study period. The effect of CaCl$_2$ on the reduction of shoot necrosis was also tested. CaCl$_2$ showed a significant (p = 0.001) effect on reducing the shoot necrosis. The lowest occurrence of shoot necrosis (25.0 %) was observed in 4.0 mM CaCl$_2$ treatment. Continuous sub-culturing of shoots with initial signs of shoot necrosis to elevated CaCl$_2$ levels until the rooting stage facilitated the recovery. Transfer of the shoots frequently into a fresh medium was not beneficial for the suppression of necrosis. Shoots maintained in the medium enriched with 4.0 mM CaCl$_2$ were transferred for acclimatisation, and this is the first report of transferring haploid coconut plants to acclimatisation conditions. The rooted shoots produced through the optimised protocol were acclimatised successfully. The prevention of shoot loss due to shoot necrosis will be beneficial for further refinement of the coconut anther culture protocol.

Keywords: Anther culture, calcium chloride, carbon source, regeneration, shoot necrosis.

INTRODUCTION

Coconut plays a vital role in the economy of tropical countries such as the Philippines, Indonesia, India, and Sri Lanka. Genetic improvement of coconut for high yield and other desirable traits is a priority research area for uplifting the coconut industry. Due to the long life span and high heterozygosity, coconut breeding through conventional methods is a long, difficult and expensive process (Nguyen *et al.,* 2015). Moreover, the production of true hybrids is hampered by high heterozygosity of coconut palm. At present, coconut breeding is done either by mass selection or crossing between varieties that have high variation within a population (Batugal *et al.*, 2009). Thus, the resultant progenies are not true hybrids. Alternative approaches to produce homozygous pure lines in coconut are highly desirable in order to improve coconut plantations. The production of double haploids (DH) is the fastest route to initiate homozygosity in plants and has been experimented with a large number of crop species (Dunwell, 1985; Abdollahi & Rashidi, 2018; Bhatia *et al*., 2018). DHs are produced by doubling the chromosomes of haploid plants, which can occur spontaneously or by chemical treatments, resulting in individuals with two identical copies of each chromosome (Dunwell, 1985).

In higher plants, doublehaploids can be introduced either through androgenesis (anther or microspore culture) or gynogenesis (ovary or megaspore culture). The availability of a few mega spores and the difficulty of fine-dissection of gametes hinder the use of mega spores for haploid plant production (Rajcan *et al.*, 2011).

The development of an effective protocol for DHs and its further application in breeding programmes is the only practical alternative for enhancing the coconut breeding strategy for the production of true hybrids. This could reduce the time required for the development of homozygous populations. Anther culture has been reported by Perera *et al*. (2008). Some of the critical factors that are required to induce microspore embryogenesis such as culture medium, pollen developmental stage, stress pre-treatment, and anther density have been discussed (Nguyen *et al*., 2015; Bandupriya *et al*., 2016). The problems associated with shoot necrosis and shoot death during in vitro culture limit further growth of plantlets up to acclimatisation stage. Moreover, the low frequency of microspore-derived embryo induction and poor plant regeneration restrict the use of anther culture technique in further developments. Thus, the use of boosted $CaCl_2$ concentrations in the regeneration medium and frequent sub-culturing was tested for overcoming shoot necrosis. Further, attempts were made to improve the anther culture protocol by studying the effect of different carbon sources on androgenic induction and of 6-benzylaminopurine (BAP) on plant regeneration efficiency.

METHODOLOGY

Plant material and explant preparation

Rachilla was collected from inflorescences of the variety 'Sri Lanka Tall' at three weeks before the splitting (3WBS) stage as described by Perera *et al*. (2008), from an adult coconut palm growing at Bandirippuwa Estate, Lunuwila, Sri Lanka. At this stage, anthers contain pollen grains at thelate uni-nucleate stage (Perera *et al.*, 2008). The middle portion of each rachillae (containing male flowers) were wrapped in aluminium foil and given a heat shock at 38 ºC for 6 d. Pre-treated anthers were excised from the male flowers and pooled anthers were surface sterilised using 2.0 % (v/v) commercial bleach (Clorox®) solution with a few drops of liquid detergent for 10 min, followed by four rinses with sterilised distilled water under aseptic conditions.

Effect of carbon source on androgenesis induction

Culture initiation and regeneration were based on the methods described by Perera *et al*. (2009) with modifications. Modified Eeuwens Y_3 medium (Eeuwens, 1976) was used in all steps until plants were transferred to the soil. A medium consisted of 100 µM 2, 4-dichlorophenoxyacetic acid (2,4-D) and 100 µM Naphthaleneacetic acid (NAA) was used as the androgenesis induction medium. The effect of the type and concentration of the carbon source on androgenesis induction was studied by culturing the pre-treated anthers into solidified media supplemented with sucrose, maltose and glucose at concentrations of 40, 90, 120 and 150 gL^{-1}. After adjusting the pH to 5.8, activated charcoal (Heycarb, Sri Lanka) at a concentration of 0.1 % (w/v) and phytagel 0.25 % (w/v) were added to the medium and autoclaved at 121ºC for 20 min. Fifteen anthers were cultured (abaxial side up) per Petri plate (90 × 18 mm) each containing 40.0 mL of culture medium. Five Petri plates were used for each treatment. The Petri plates were incubated in the dark at 27 ± 1 ºC until embryos emerged. The number of anthers that produced embryos was counted and recorded after 08 months from culture initiation.

Effect of BAP on regeneration

The embryos were sub-cultured into somatic embryo induction medium with reduced 2,4-D (70.0 µM) solidified with 0.25 % (w/v) phytagel, followed by maturation medium devoid of any growth regulators and solidified with 0.30 % (w/v) phytagel. Mature embryos were collected, bulked and cultured on the germination medium supplemented with different concentrations of BAP (5.0, 10.0, 20.0, and 25.0 µM) for further proliferation and shoot initiation. Three sub cultures were added into the same fresh medium until shoots emerged. Well-developed germinating embryos were then transferred to regeneration medium supplemented with 0.45 µM gibberellic acid (GA_3). Cultures were maintained for 6 wks in each media mentioned above before being transferred to the next medium. Finally, continuous sub-culturing was done at 6-week intervals (unless otherwise stated) into fresh GA_3 containing media until shoots developed. All culture media contained 0.1 % (w/v) activated charcoal. The cultures were maintained in the dark at 27 ± 1 ºC until the embryos germinated. The germinated embryos (with shoot sprouts) were then exposed to 16 h photoperiod (PAR; 25 µmolm-2s⁻¹). The number of embryos converted into shoots was counted. Shoots longer than 1.5 cm was counted in each treatment after 08 months from the first culture of embryos into BAP containing medium. The experiment was repeated three times.

Rooting and acclimatisation of anther cultured plants

Regenerated shoots were transferred to a medium

containing 0.5 µM indole acetic acid (IAA) to induce rooting. Once the roots developed, the plantlets were transferred to a liquid medium supplemented with the same concentration of IAA. Plants were maintained in a medium supplemented with high $CaCl_2$ (4.0 mM) until they were transferred to the soil medium for acclimatisation. Plants with 3–4 well-developed leaves and a healthy root system (Figure 3c) were carefully removed from the liquid medium and each plant was transferred to a propagator containing a potting mixture of sand, soil, and coir dust (1:1:1).

Reduction in shoot necrosis

Two methods were tested for the reduction of shoot necrosis at shoot multiplication stage. To determine the effect of $CaCl_2$ on shoot necrosis, different levels of $CaCl_2$ (2.0, 3.0, and 4.0 mM) were incorporated into germination medium and the same levels were maintained until plants were transferred to soil. Twenty germinating embryos were used for each treatment and the number of plants showing necrosis in each treatment was recorded.

Sub-culturing the shoots into a fresh shoot multiplication medium at 3-wk intervals instead of 6 wks was also attempted. A new set of anther-derived shoots was used for this experiment. Eight shoots were used for each treatment and the experiment was carried out twice.

Experimental design and data analysis

The experiment was designed as a two-factor factorial laid on a completely randomised design (CRD) to determine the effect of carbon source on androgenesis induction. Three sugar types and four concentrations were considered as factors. The experiment was repeated three times. Percentage of embryo production data were analysed using two-way ANOVA after confirming the normality of the data with Anderson Darling normality test (AD = 0.622, p = 0.093). Post-hoc evaluations were done with Tukey's test to find the best sugar type with the correct concentration combination.

The experiment to determine the effect of four BAP concentrations on shoot regeneration was designed as a simple CRD experiment with three replicates. The percentage of embryos converted into shoots and the percentage of embryos that produced shoots longer than 1.5 cm were tested for normality with Anderson Darling test, and one-way ANOVA was used for data analysis.

Elucidating the effect of $CaCl_2$ on the reduction of shoot necrosis was done using three different $CaCl_2$ concentrations in a simple CRD experiment. Twenty germinating embryos were used in each treatment.

Binary logistic models were used to compare the probability of shoot necrosis (as it is a binary response) based on the 03 $CaCl_2$ concentrations as a categorical predictor and to compare the effect of the number of subcultures on necrosis.

RESULTS AND DISCUSSION

Androgenesis was successfully induced in cultured anthers of coconut (Figure 1a – d). Shoots emerged either through a germination point in the embryo or by splitting the haustorial tissue. Single or multiple shoots were successfully developed into complete plantlets.

### Effect of type of sugar and concentration on embryo production

Androgenesis efficiency was determined based on the percentage number of embryos produced in cultured anthers under different sugar treatments. The results revealed that the concentration and the type of sugar and their interaction indicate the effect of sugar type on the level of embryo production, is dependent on the sugar concentration.
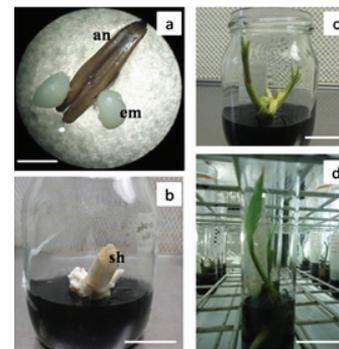
Figure 1: Plant regeneration through coconut anther culture. a - embryos (em) derived from anthers (an) after three months of culture initiation (Bar 2 mm); b - germinating embryo, the emerging shoot (Sh) through a germination point in the embryo (Bar 2.0 cm); c - multiple shoots developed from a single embryo (Bar 2.5 cm); d - complete plantlet (Bar 4.0 cm)

Embryo production was promoted in all four sucrose concentrations. Post hoc evaluation of the interaction effect revealed that the 90 gL^{-1} sucrose concentration performed superior to the other sugar treatments (Figure 2) and recorded the highest percentage of embryo production (44.0 %, Figure 2). There was a reduction in embryo production with the increase of sucrose concentration in the medium. The incorporation of maltose instead of sucrose showed a reduction in embryo production when maltose was added either as 40.0 gL^{-1} or 90.0 gL^{-1} concentration. Maltose added at a concentration of 120.0 gL^{-1} produced significantly higher embryo production equal to 90.0 gL^{-1} sucrose. The effect of higher concentrations (especially 150.0 gL^{-1}) on embryo production was found to be unfavourable and showed a reduced embryo production in both sucrose and maltose. Interestingly at 120.0 gL^{-1} concentration, both sucrose and maltose showed a similar production of embryos. In general, glucose at all concentrations showed the least production of embryos (Figure 2).
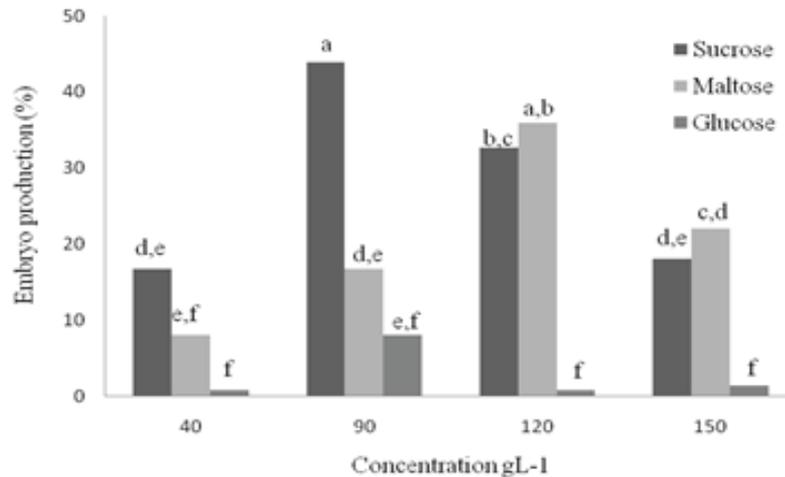
Figure 2: Effect of different sugar types and concentrations on androgenic responses of coconut anthers in androgenesis induction medium. Percentages (mean) with different letters of each parameter are significantly different ($p < 0.05$).

Effect of BAP on shoot regeneration

Results revealed that the different concentrations of BAP act significantly on embryos to generate shoots (F = 16.5, p = 0.001) and to produce healthy shoots (longer than 1.5 cm) eight months after embryos are transferred to germination medium (F = 9.22, p = 0.006).

Out of the concentrations used in the study, 20.0 µM BAP showed significantly high shoot generation as well as significantly longer shoots during the study period (Table 1). The percentage of embryos converted to shoots in the 20.0 µM BAP concentration was threefold compared to the control medium supplemented with 5.0 µM. Further increase of BAP concentration reduced the conversion of embryos into shoots (Table 1).

Table 1: Effect of different BAP concentrations in the germination medium on plant regeneration in anther culture of coconut

Concentration of BAP (µM)	Percentage embryos converted to produce shoots*	Percentage embryos with shoots longer than 1.5 cm*
5.0	15.74 [B]	07.87 [B]
10.0	19.91 [B]	15.74 [B]
20.0	47.62 [A]	31.75 [A]
25.0	27.78 [B]	19.91 [AB]

[A,B] Means with the same letters along the columns are not significantly different at $p < 0.05$ at 95 % confidence level
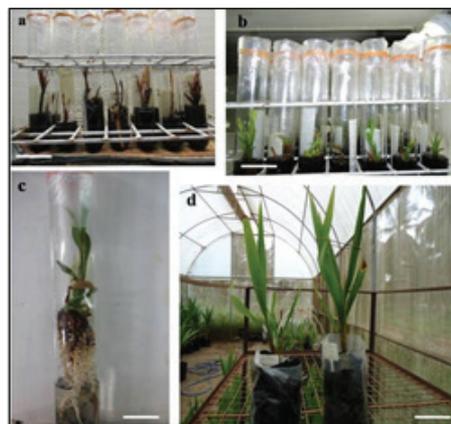
Effect of CaCl$_2$ on shoot necrosis

Shoots derived through androgenesis and raised in regeneration medium [supplemented with normal CaCl$_2$ (2.0 mM)] were affected by shoot necrosis and eventually died (Figure 3a). The symptoms started at either in leaves or immature stem in almost all the cultures. This serious problem made it difficult to raise plantlets up to acclimatisation stage. In order to elucidate the effect of CaCl$_2$ on the reduction of shoot necrosis, shoots derived from coconut anthers were cultured in the germination medium supplemented with elevated CaCl$_2$ concentrations. The germination medium used in this particular experiment comprised the best BAP (20.0 μM) concentration, which was determined in a previous experiment. It was found that CaCl$_2$ can cause a significant effect on shoot necrosis (G-square = 13.63, p = 0.001). The results of goodness-of-fit tests (Deviance, Pearson & Hosmer-Lemeshow) are all greater than the significance level of 0.05 (Chi-Square = 0.64, p = 0.72), which indicates that the use of binary logistic models is appropriate.

Table 2: The effect of CaCl$_2$ concentration on the reduction of shoot necrosis

CaCl$_2$ Concentration (mM)	Percentage necrosis Mean ± SE
2.0	84.21 ± 8.59^A
3.0	66.7± 12.6^A
4.0	25.00 ± 11.2^B

^{A,B} Means with the same letters along the columns are not significantly different at p < 0.05 at 95 % confidence level

According to the results presented in Table 2, plantlets treated with 2.0 mM CaCl$_2$ concentration showed significantly high necrosis than with 4.0 mM treatment. Three millimolar (3.0 mM) CaCl$_2$ and 4.0 mM CaCl$_2$ concentrations also showed statistical significance for having different levels of necrosis in respective cultures. However, there was no significantly different occurrence of necrosis between the CaCl$_2$ concentrations 2.0 mM and 3.0 mM. The highest level of shoot necrosis (84.21 %) was observed in the medium containing 2.0 mM CaCl$_2$, which is the concentration present in the normal Y$_3$ medium used in routine culturing.

Figure 3. The effect of CaCl$_2$ concentration on the reduction of shoot necrosis. a – dying shoots in the regeneration medium (supplemented with 2.0 mM CaCl$_2$) due to shoot necrosis (Bar 4.0 cm); b - healthy shoots in regeneration medium supplemented with 4.0 mM CaCl$_2$ (Bar 3.0 cm); c - complete plantlet in the rooting medium (Bar 2.0 cm); d - plants at acclimatisation stage (Bar 6.0 cm)

Continuous sub-culturing of the shoots with initial signs of shoot necrosis into media supplemented with 4.0 mM CaCl$_2$ until they developed a good root system has facilitated the recovery. Shoots maintained in the medium enriched with 4.0 mM CaCl$_2$ were transferred for acclimatisation (Figure 3c, 3d). This is the first report of transferring coconut plants developed through androgenesis into acclimatisation conditions.

Effect of number of subcultures on necrosis

Subculture of necrotic shoots into shoot multiplication medium at three-week intervals instead of six weeks did not significantly alleviate the problem of necrosis. It was revealed (Table 3) that there is no significant relationship between the number of subcultures and the occurrence of necrosis (Chi sq. = 0, p = 1.00). The odds ratio 1 indicated that both levels have a similar chance of occurrence of necrosis.

Table 3: Effect of sub-culturing on shoot necrosis reduction.

Subculture interval (weeks)	% shoot necrosis ± SD
3	56.25 ± 06.25
6	56.25 ± 18.75

Carbohydrate that acts as the source of carbon and energy during plant regeneration is a common, important component in coconut tissue culture media (Nguyen *et al*., 2015). Although sucrose is reported as the common sugar type in many of the plant tissue culture media (Yaseen *et al.*, 2013), other sugars such as maltose, glucose and some tri-saccharides and pentoses have the potential to metabolise during androgenesis (Yaseen *et al.*, 2013). According to the results obtained in the present study, the carbohydrate source is one of the major components that support the conversion of anthers into embryos. Both, the type of carbohydrate and carbohydrate concentration, affect the results obtained for the tested parameters. Out of the carbohydrates tested, sucrose and maltose were superior to glucose. A sucrose concentration of 90.0 gL^{-1} and maltose concentration of 120.0 gL^{-1} showed significantly higher ($p < 0.05$) embryo (44.0 % and 36.0 %, respectively) production. Similar results of using high concentrations of sucrose have been reported elsewhere showing significantly higher embryo production in maize (Buter, 1997). Sucrose is an easily metabolised sugar, which shows a variety of effects on plant cell and tissue culture (Vitova *et al.,* 2002). It is reported that sucrose can control the expression of pathogenesis-related genes in plants (Herbers *et al.*, 1996). Moreover, it has been reported that high concentrations of carbohydrates improve embryogenesis by creating an osmotic stress (Agarwal *et al.,* 2004). In addition under osmotic stress conditions, polyamine synthesis in plant cells increases causing favourable conditions for embryogenesis (Litz, 1986). However, sucrose levels higher than 90.0 gL^{-1} showed an inhibitory effect on coconut anthers. Higher levels of sucrose (150.0 gL^{-1}) adversely affected the production of embryos (Figure 2). Similar observations on the reduction of embryo production upon elevated sucrose levels have been reported for species such as barley (Marsolais & Kasha, 1985), rye (Guo & Pulli, 2000), and *Cucumis sativus* (Ashok & Murthy, 2004).

Maltose has been superior to sucrose as a carbohydrate source for androgenesis in several species including cereals. Culturing of barley microspores in media supplemented with sucrose, glucose, or fructose was found to be deleterious, whereas maltose acted favourably on embryo production (Scott & Lyne, 1994). The capacity of barley microspores to differentiate and induce green plantlets has been enhanced by both maltose and malt extracts (Finnie *et al*., 1989). The effect has been determined in relation to the osmotic regulation of microspores during the induction phase (Sunderland & Dunwell, 1977). However, in the present study maltose effect did not surpass the same observed in sucrose but showed similar results with sucrose at a comparatively higher concentration of maltose.

The effect of different concentration regimes of BAP on the conversion and further proliferation of embryos into shoots was investigated. BAP is considered as a chemically stable cytokinine in plant tissues and it is the commonly preferred cytokinine by plant tissue culturists (Klems *et al*., 2000). Initial work on coconut androgenesis has shown that conversion of embryos into plantlets is possible in the presence of 5.0 µM BAP (Perera *et al.*, 2008; 2009). However, extremely low percentage (7.0 %) of shoot conversion has been reported. In a recent study, Perera *et al.* (2020) reported that BAP concentration plays a significant role in converting anther derived embryos into shoots. Maximum embryo sprouting has been reported in the media supplemented with 25.0 and 35.0 µm BAP with a record of 50.0 % and 53.0 % conversions, respectively. However, the greatest shoot development recorded in the study conducted by Perera *et al.* (2020) was less than 30.0 %, in the medium supplemented with 35.0 µm BAP. Nevertheless, in the present study, nearly 50.0 % of the embryos were converted into plantlets when 20.0 µM BAP was used. The use of modified Y_3 medium formulated specifically for coconut in vitro culture instead of Murashige and Skoog medium is one of the major differences between these two studies. Moreover, different culture incubation durations in BAP incorporated media were maintained in these two studies.

Hormones usually tend to show the maximum shooting response at its optimum concentration. Farahani *et al.* (2008) reported that the shoot multiplication of *Musa acuminata* was affected by the concentration of BAP. Later in 2015, Ferdous *et al.* revealed the maximum single shoot formation and longest shoot formation in M. acuminata at 0.5 mg/L BAP. Similarly, determination of the precise concentration of BAP for maximum shoot regeneration has been reported in several other studies in different crop plants. Gubi *et al.* (2004), Kadota and Niimi (2003), Klems *et al.* (2000) and Jafari *et al.* (2011) reported that overexposure of cultures to higher concentrations of BAP might lead to hyperhydric shoots, which was not observed in the present study. However, Katoda and Niimi (2003) reported that the occurrence of hyperhydric cultures is less in BAP supplemented culture media when compared to media incorporated with synthetic cytokinines such as *N*-(2-chloro-4-pyridyl)-N9-phenylurea (CPPU) and 1-phenyl-3- (1,2,3-thiadiazol-5-yl) urea (TDZ). Shoots consisting of single, double or multiple (Figure 1c) shoots were produced in BAP

supplemented media, which is in accordance with the previous reports of coconut androgenesis (Perera *et al.*, 2009). Ploidy analysis of coconut anther-derived plants has been performed previously on the current protocol by flow cytometry analysis and recorded a high double haploid yield (Perera *et al.*, 2008). Thus, ploidy analysis studies were not executed for the current study since similar conditions were used as in the previous study.

Shoot necrosis is one of the obstacles associated with the androgenesis of coconut. The survival of shoots regenerated through androgenesis was difficult due to high shoot necrosis. An increase in calcium concentration in the regeneration medium from 2.0 mM to 4.0 mM has recorded higher recovery of anther-derived shoots affected by shoot necrosis, and reduced shoot necrosis from 84.21 % to 25.00 %. Similar results have been reported in *vitro* for several other perennial crops such as grapes (Surakshitha *et al.*, 2019), oak (Vieitez *et al.*, 1989), banana (Martin *et al.*, 2007) and *Trichosantes dioica* (Kishore *et al.*, 2015). As discussed in previous reports, the occurrence of necrosis in coconut shoots developed through androgenesis may be associated with the calcium deficiency. Calcium is a major nutrient required for plant growth and it is responsible for the growth and differentiation of plant cells, formation of the cell wall, maintain membrane permeability and (Hepler, 2005; Stael *et al.*, 2012). Thus, calcium deficiency in plant tissues could disturb metabolic activities in developing tissues, and as such, metabolic imbalances could be visualised as growth abnormalities like shoot necrosis (Surakshitha *et al.*, 2019). According to Hirschi (2004), upward movement of calcium ion in the xylem sap is basically due to an efficient transpiration system. Therefore, it is suggested that conditions existing in the culture vessels that limit an efficient transpiration stream may be another way by which the mobility of calcium ions is limited in the in vitro plantlets, causing deficiency symptoms such as shoot necrosis. Since two times of the normal Ca^+ concentration of the Y_3 medium was sufficient to reduce shoot necrosis, the effect may have caused low or no effect on shoot necrosis in this particular situation. The recovery or low necrosis in plantlets in high Ca^{+2} ion medium may be due to enhancing the mitotic process, possibly by regulating other hormonal signalling functions as described by Hepler and Wayne (1985). Bairu et al. (2009) reported that elevated BAP levels increased the occurrence of shoot necrosis in *Harpagophytum procumbens.*

However, mitigation of shoot necrosis was possible in the present study even at a higher BAP (20.0 μM) concentration, when a Ca^{+2} rich medium was used.

Plantlets recovered in the Ca^{+2} rich medium were successfully acclimatised and subjected to greenhouse conditions (Figure 3d). Androgeneis is a highly genotype dependent activity (Bhatia *et al.*, 2017). Since the modified protocol described above was developed for a Tall coconut variety, which is a cross pollinating variety, the successful application of adrogenesis in this variety will enable successful coconut breeding. When androgenesis protocols are being developed for other coconut varieties, the above mentioned facts could be considered to develop better protocols.

CONCLUSIONS

In conclusion, the present study demonstrated that manipulating different stages of androgenesis process in vitro could enhance the plantlet production up to a considerable level. Successful embryo production was achieved in sucrose and maltose when applied at 90.0 gL^{-1} or 120.0 gL^{-1}, respectively. Twenty micromolar (20 μM) BAP showed the best shoot regeneration in anther derived embryos. The increase of $CaCl_2$ concentration significantly affected alleviation of the problem of necrosis. Transfer of shoots at very early stages and continuous subculture of shoots into high $CaCl_2$ containing (4.0 mM) regeneration medium effectively reduced the occurrence of necrosis.

Conflicts of interest:

The authors have declared that there is no conflict of interest.

REFERENCES

Abdollahi M.R. & Rashidi S. (2018). Production and conversion of haploid embryos in chickpea (*Cicer arietinum* L.) anther cultures using high 2,4-D and silver nitrate containing media. *Plant Cell Tissue and Organ* Culture **133**: 39–49. DOI: https://doi.org/10.1007/s11240-017-1359-4

Agarwal S., Kanwar K. & Sharma D.R (2004). Factors affecting secondary somatic embryogenesis and embryo maturation

in *Morus alba* L. *Scientia Horticulturae* **102**: 359–368.
DOI: https://doi.org/10.1016/j.scienta.2004.04.002

Ashok K.H.G. & Murthy H.N. (2004). Effects of sugars and amino acids on androgenesis of *Cucumis sativus* L. *Plant Cell Tissue and Organ Culture* **78**: 201–208.
DOI: https://doi.org/10.1023/B:TICU.0000025637.56693.68

Bairu M.W., Jain N., Stirk W.A, Dolezal K. & Staden J.V. (2009). Solving the problem of shoot-tip necrosis in *Harpagophytum procumbens* by changing the cytokinin types, calcium and boron concentrations in the medium. *South African Journal of Botany* **75**: 122–127.
DOI: https://doi.org/10.1016/j.sajb.2008.08.006

Bandupriya H.D.D., Femando S.C. & Vidhanaarachchi V.R.M. (2016). Micropropagation and androgenesis in coconut: an assessment of Sri Lankan implication. *Cocos* **22**: 31–47.
DOI:https://doi.org/10.4038/cocos.v22i1.5810

Batugal P., Bourdeix R. & Baudouin L. (2009). Coconut breeding. In: *Breeding Plantation Tree Crops: Tropical Species* (eds. S.M. Jain & P.M. Priyadarshan), pp. 327–375. Springer, New York, USA.
DOI:https://doi.org/10.1007/978-0-387-71201-7_10

Bhatia R., Dey S.S., Parkash C., Sharma K., Sood S. & Kumar R. (2018). Modification of important factors for efficient microspore embryogenesis and doubled haploid production in field grown white cabbage (*Brassica oleracea* var. capitata L.) genotypes in India. *Scientia Horticulturae* **233**: 178–187.
DOI: https://doi.org/10.1016/j.scienta.2018.01.017

Bhatia R., Dey S.S., Sood S., Sharma K., Parkash C. & Kumar R. (2017). Efficient microspore embryogenesis in cauliflower (*Brassica oleracea* var. botrytis L.) for development of plants with different ploidy level and their use in breeding programme. *Scientia Horticulturae* **216**: 83–92.
DOI: https://doi.org/10.1016/j.scienta.2016.12.020

Buter B. (1997). In *vitro* haploid production in maize. In: *In vitro Haploid Production in Higher Plants* (eds. S. Mohan Jain, S.K. Sopory & R.E. Veilleux), pp 37–71. Kluwer Academic Publishers, Dordrecht, The Netherlands.
DOI: https://doi.org/10.1007/978-94-017-1862-2_2

Dunwell J. M. (1985). Anther and ovary culture. In: *Cereal Tissue and Cell Culture* (eds. S.W.J. Bright & M.G.K. Jones), pp. 1–44. Matinus Nijhoff/ Dr. W. Junk Publishers, Dortrecht, The Netherlands.
DOI: https://doi.org/10.1007/978-94-009-5133-4_1

Eeuwens C.J. (1976). Mineral requirements for growth & callus initiation of tissue explants excised from mature coconut palm (*Cocos nucifera* L.) cultured in *vitro*. *Physiologia Plantarum* **36**: 23–28.
DOI: https://doi.org/10.1111/j.1399-3054.1976.tb05022.x

Farahani F., Aminpoor H., Sheidai M., Noormohammadi Z. & Mazinani M.H. (2008). An improved system for in vitro propagation of banana (*Musa acuminate* L.) cultivars. *Asian Journal of Plant Sciences* **7**: 116–118.
DOI:https://doi.org/10.3923/ajps.2008.116.118

Ferdous M.H., Billah A.A.M., Mehraj H., Taufiqu T. & Uddin A.F.M.J. (2015). BAP and IBA pulsing for in vitro multiplication of banana cultivars through shoot-tip culture. *Journal of Biosciences and Agricultural Research* **3**: 87–95.
DOI:https://doi.org/10.18801/jbar.030215.35

Finnie S.J., Powell W. & Dyer A.F. (1989). The effect of carbohydrate composition and concentration on anther culture response in barley (*Hordeum vulgare* L.). *Plant Breeding* **103**: 110–118.
DOI: https://doi.org/10.1111/j.1439-0523.1989.tb00358.x

Gubi J., Lajchova Z., Farago J. & Jurekova Z. (2004). Effect of growth regulators on shoot induction and plant regeneration in tomato (*Lycopersicon esculentum* Mill.). Biologia **59**: 405–408.

Guo Y.D. & Pulli S. (2000). Isolated microspore culture and plant regeneration in rye (*Secale cereale* L.). Plant *Cell Reports* **19**: 875–880.
DOI: https://doi.org/10.1007/s002990000194

Hepler P.K. (2005). Calcium: a central regulator of plant growth and development. *Plant Cell* **17**: 2142–2155.
DOI: DOI: https://doi.org/10.1105/tpc.105.032508

Hepler P.K. & Wayne R.O. (1985). Calcium and plant development. *Annual Review of Plant Physiology* **36**: 397–439.
DOI:https://doi.org/10.1146/annurev.pp.36.060185.002145

Herbers K., Meuwly P., Frommer W.B., Métraux J.P. & Sonnewald U. (1996). Systemic acquired resistance mediated by the ectopic expression of invertase: possible hexose sensing in the secretory pathway. *Plant Cell* **8**: 793–798.
DOI: https://doi.org/10.1105/tpc.8.5.793

Hirschi K.D. (2004). The calcium conundrum. Both versatile nutrient and specific signal. *Plant Physiology* **136**: 2438–2442.
DOI: https://doi.org/10.1105/tpc.8.5.793

Jafari N., Othman R.F. & Khalid K. (2011). Effect of benzylaminopurine (BAP) pulsing on in vitro shoot multiplication of *Musa acuminata* (banana) cv. Berangan. *African Journal of Biotechnology* **10**: 2446–2450.

Kadota M. & Niimi Y. (2003). Effect of cytokinin types and their concentration on shoot proliferation and hyperhydricity in in *vitro* pear cultivar shoots. *Plant Cell Tissue and Organ Culture* **72**: 261–265.
DOI: https://doi.org/10.1023/A:1022378511659

Kishore K., Patnaik S. & Shukla A. K. (2015). Optimization of method to alleviate in *vitro* shoot tip necrosis in Trichosantes dioica Roxb. *Indian Journal of Biotechnology* **14**: 107–111.

Klems M.J., Balla J., Machackova I., Eder J. & Prochazka S. (2000). The uptake and metabolism of H-3-benzylaminopurine in tobacco (*Nicotiana tabacum* L.) and cucumber (*Cucumis sativus* L.) explants. *Plant Growth Regulation* **31**: 135–142.
DOI: https://doi.org/10.1023/A:1006374611228

Litz R.E. (1986). Effect of osmotic stress on embryogenesis in Carica suspension culture. *Journal of the American Society for Horticultural Science* **111**: 969–972.

Marsolais A.A. & Kasha K.J. (1985). The role of sucrose and auxin in a barley anther culture medium. *Canadian Journal*

of Botany **63**: 2209–2212.
DOI:https://doi.org/10.1139/b85-313

Martin K.P., Zang C.L., Slater A. & Madassery J. (2007). Control of shoot necrosis and plant death during micropropagation of banana and plantain (*Musa* spp.). *Plant Cell Tissue and Organ Culture* **88**: 51–59.
DOI: https://doi.org/10.1007/s11240-006-9177-0

Nguyen Q.T., Bandupriya H.D.D., Villalobo A.L., Sisunandar S., Foale M. & Adkins S.W. (2015). Tissue culture and associated biotechnological interventions for the improvement of coconut (*Cocos nucifera* L.): a review. Planta **242**: 1059–1076.
DOI: https://doi.org/10.1007/s00425-015-2362-9

Perera P.I.P., Hocher V., Verdeil J-L., Bandupriya H.D.D., Yakandawala D.M.Y. & Weerakoon L.K. (2008). Androgenic potential of coconut (*Cocos nucifera* L.). *Plant Cell Tissue and Organ Culture* **92**: 293–302.
DOI: https://doi.org/10.1007/s11240-008-9337-5

Perera P.I.P., Motha K.H. & Vidhanaarchchi V.R.M. (2020). Morphological and histological analysis of anther-derived embryos of coconut (*Cocos nucifera* L.). *Plant Cell Tissue and Organ Culture* **140**: 685–689.
DOI: https://doi.org/10.1007/s11240-019-01762-9

Perera P.I.P., Yakandawala D.M.Y., Hocher V., Verdeil J-L. & Weerakoon L.K. (2009). Effect of growth regulators on microspore embryogenesis in coconut anthers. *Plant Cell Tissue and Organ Culture* **96**: 171–180.
DOI: https://doi.org/10.1007/s11240-008-9473-y

Rajcan I., Boersma J.G. & Shaw E.J. (2011). Plant genetic techniques: plant breeder's toolbox. In: *Comprehensive Biotechnology*, second edition (ed. M.M. Young), pp. 133–147. Academic Press, USA.

DOI::// https doi.org/10.1016/B978-0-08-088504-9.00252-X

Scott P. & Lyne R.L. (1994). The effect of different carbohydrate sources upon the initiation of embryogenesis from barley microspores. *Plant Cell Tissue and Organ Culture* **36**: 129–133.
DOI: https://doi.org/10.1007/BF00048323

Stael S., Wurzinger B., Mair A., Mehlmer N., Vothknecht U.C. & Teige M. (2012). Plant organellar calcium signalling: an emerging field. *Journal of Experimental Botany* **63**: 1525–1542.
DOI: https://doi.org/10.1093/jxb/err394

Sunderland N. & Dunwell J.M. (1977). Anther and pollen culture In: *Plant Tissue and Cell Culture* (ed. H.E. Street), pp. 223–265, Botany Monographs volume 11. Blackwell Scientific Publications. Oxford, London.

Surakshitha N.C., Soorianathasundaram K., Ganga M. & Raveendran M. (2019). Alleviating shoot tip necrosis during *in vitro* propagation of grape cv. Red Globe. *Scientia Horticulturae* **248**: 118–125.
DOI: https://doi.org/10.1016/j.scienta.2019.01.013

Vieitez A.M., Sanchez C. & San-Jose C. (1989). Prevention of shoot tip necrosis in shoot cultures of chestnut and oak. *Scientia Horticulturae* **41**: 101–109.
DOI: https://doi.org/10.1016/0304-4238(89)90059-9

Vitova L., Stodulkova E., Bartonickova A. & Lipavska H. (2002). Mannitol utilisation by celery (Apium graveolens) plants grown under different conditions *in vitro*. *Plant Science* **163**: 907–916.
DOI: https://doi.org/10.1016/S0168-9452(02)00240-6

Yaseen M., Ahmad T., Sablok G., Standardi A. & Hafiz I.A. (2013). Role of carbon sources for in vitro plant growth and development. *Molecular Biology Reports* **40**: 2837–2849.
DOI: https://doi.org/10.1007/s11033-012-2299-z