

## RESEARCH ARTICLE

# Effect of genotype, embryo maturity and culture medium on *in vitro* embryo germination of Sri Lankan coconut (*Cocos nucifera* L.) varieties

V.R.M. Vidhanaarachchi<sup>1\*</sup>, W.C. Suranjith<sup>2</sup> and T.R. Gunathilake<sup>1</sup>

<sup>1</sup> Tissue Culture Division, Coconut Research Institute, Bandirippuwa Estate, Lunuwila.

<sup>2</sup> Quality Assurance Department, CBL Foods International Pvt. Ltd., Habarakada Road, Ranala.

Revised: 13 October 2015; Accepted: 30 December 2015

**Abstract:** Poor *in vitro* germination of embryos of exotic varieties was reported as one of the major constraints faced during coconut germplasm exchange programmes. In this study, the effect of genotype, embryo maturity and culture medium on *in vitro* germination of coconut embryos was investigated. A significant effect ( $p < 0.05$ ) of genotype on *in vitro* germination was observed between the selected cultivars, San Ramon Tall (SNRT) (77.48 %), Sri Lanka Red Dwarf (SLRD) (67.28 %), Sri Lanka Green Dwarf (PGD) (71.85 %) and King Coconut (RTB) (52.5 %). Embryo germination percentages were improved in solid media (91.66 % and 92.22 % in 75 g/L and 60 g/L sucrose respectively,  $p < 0.05$ ) than in liquid media (56.66 % and 60.46 % in 75 g/L and 60 g/L sucrose, respectively) and the sucrose concentration has no effect on germination of SLRD embryos. Furthermore, maturity of the nut significantly affected the germination ( $p < 0.05$ ) of embryos of PGD and the highest *in vitro* embryo germination was achieved by culturing embryos of the 12 month old bunch (97.67 %), while the lowest was observed in the 10 month old bunch (52.17 %). Addition of growth hormones favoured root growth of *in vitro* raised SLRD plants with a significant increase in the number of primary roots and the number of leaves ( $p < 0.05$ ) when 10  $\mu$ M BAP, 10  $\mu$ M Kinetin and 200  $\mu$ M NAA were added to the embryo culture medium.

**Keywords:** Coconut, cultivars, embryo culture, *in vitro* embryo germination, shoot and root growth.

## INTRODUCTION

Conservation of coconut genetic resources is of vital importance for present and future breeding programmes for maintaining the genetic diversity (Weerakoon *et al.*,

2002). Based on loss of palms, the rate of reduction of the existing coconut genotypes in Sri Lanka was estimated to be around 2 % annually, leading to genetic erosion. The main causes of genetic erosion were land fragmentation for urbanisation/industrialisation and replacement of locally adapted old coconut plantations with improved coconut cultivars, which has a narrow genetic base. Biotic and abiotic factors such as drought and cyclones have also been identified as causes of the genetic erosion to a lesser extent. Therefore, systematic collection and conservation of coconut germplasm at the Coconut Research Institute, Sri Lanka (CRISL) was initiated in 1984 in order to conserve the existing germplasm and to obtain locally adapted materials for future breeding programmes. However, DNA based germplasm characterisation studies carried out at the CRISL revealed that the genetic base of the available coconut gene pool in Sri Lanka is narrow, and therefore further improvement of coconut by utilising only locally available materials is limited (Perera *et al.*, 1999). Moreover, it was also revealed that the South-East Asia and Pacific regions have a wider genetic diversity when compared with the South Asian and African regions (Perera *et al.*, 2003). Therefore, it was decided that incorporation of exotic germplasm, especially from South-East Asia and Pacific is important for further improvement of coconut in Sri Lanka.

Coconut is one of the largest seed producing plant species within the Plant Kingdom. Quick germination can be observed after maturation, which negatively affect the collection and exchange of coconut germplasm among countries. The introduction of exotic germplasm

\* Corresponding author (vijitharma@yahoo.com)

has also been restricted by quarantine regulations considering possible inadvertent introduction of many pests and lethal diseases prevalent in other coconut growing countries.

Therefore, coconut germplasm exchange is possible only as embryos or embryo containing endosperm plugs and raising of seedlings through *in vitro* embryo culture technique, which prevent the introduction of pests and diseases inadvertently (Assy-Bah *et al.*, 1987). Mature embryos of 10 – 12 month old nuts are collected, sterilised and pre-cultured in storage medium in cryovials individually. These cryovials can be hand carried or be sent by air mail through courier services to recipient countries. Once they arrive at the recipient laboratories the embryos are transferred to normal embryo germination medium and raised to plants, acclimatised and field planted. One of the major drawbacks in germplasm exchange programmes is poor germination of embryos of some exotic genotypes, and the possible reasons identified are the effect of genotypes, maturity of nuts and long storage time during collection and transport (Cueto *et al.*, 2012). These should be minimised to achieve an acceptable level of embryo germination in order to develop a viable germplasm exchange programme. Therefore, this study was focused on evaluating the genotype effect on *in vitro* embryo germination and possible improvements to the germination medium. The effect of nut maturity on *in vitro* germination of embryos was also evaluated. In addition to the above drawbacks at the germination stage, poor root growth of *in vitro* raised seedlings is one of the reasons for low survival rates at the acclimatisation stage. Therefore, to improve root growth of *in vitro* seedlings different auxin treatments were tested with plants four (04) months after germination.

## METHODOLOGY

### Materials

Four locally grown coconut cultivars, namely, Sri Lanka Red Dwarf (SLRD), Sri Lanka Green Dwarf (PGD), San Ramon Tall (SNRT) and King Coconut (RTB) were used in this experiment. Mature nuts were harvested from 12 month old bunches unless otherwise specified, de-husked and split opened. Endosperm plugs were extracted using a cork borer. The extracted endosperm plugs were properly rinsed out 4 – 5 times in tap water after adding a few drops of liquid soap. Zygotic embryos were dissected from endosperm plugs with a scalpel blade and collected (Figure 1).

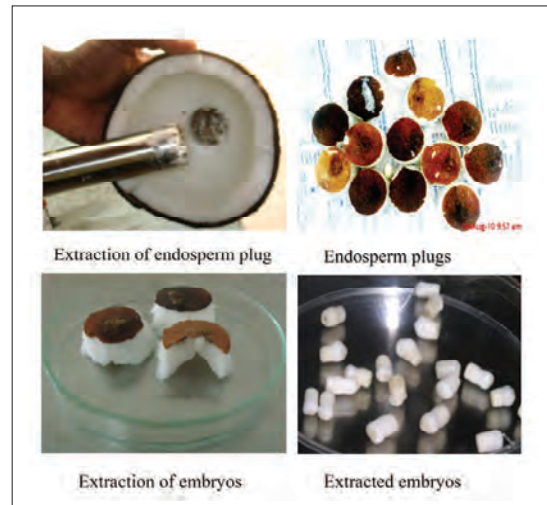


Figure 1: Collection of coconut embryos for *in vitro* culture

### Embryo culture

Surface sterilisation of the embryos was carried out using newly prepared 5 % bleach solution with a few drops of liquid soap followed by washing 4 – 5 times with sterilised distilled water. The sterilised embryos were then cultured on modified Y<sub>3</sub> medium (Karunaratne *et al.*, 1985) and incubated under dark conditions at 28 °C for 2 months. Germinated embryos were transferred to light with 16 hrs photoperiod (Temperature 28 ± 1 °C, PAR: about 75 μmol m<sup>-2</sup>s<sup>-1</sup>). Germinated embryos and plants were sub-cultured onto fresh media at every 5 – 6 week intervals.

### Effect of genotype on *in vitro* embryo germination

Embryo germination of Sri Lanka Red Dwarf (SLRD), Sri Lanka Green Dwarf (PGD), San Ramon Tall (SNRT) and King Coconut (RTB) were evaluated. Four replicates consisting of 20 embryos were used from each cultivar and modified Eeuwens Y<sub>3</sub> medium (Karunaratne *et al.*, 1985) was used as the culture medium.

### Effect of germination medium on *in vitro* embryo germination of SLRD

Modified Eeuwens Y<sub>3</sub> medium (Karunaratne *et al.*, 1985) was used as the basal culture medium with two levels of sucrose (60 g/L and 75 g/L), and solid (by adding 0.7 % agar) and liquid states of medium were combined to get four different treatments. Each treatment was replicated 6 times with 15 embryos per each replicate. Embryos of SLRD were used as the explants and embryo germination and plant development were recorded.

### Effect of embryo maturity on *in vitro* germination of PGD

Zygotic embryos were obtained from PGD to determine the most suitable maturity stage of nuts for embryo culture. Nuts were obtained from 10, 11 and 12 month old bunches (considering the last opened inflorescence as 0) and the embryos were cultured in modified Eeuwens Y<sub>3</sub> medium (Karunaratne *et al.*, 1985). Two replicates with 20 embryos per replicate were used for each treatment and embryo germination was recorded.

### Effect of hormones on root development of SLRD

*In vitro* raised plants of SLRD were used to evaluate the effect of indoleacetic acid (IAA), benzyl amino purine (BAP), kinetin and naphthaleneacetic acid (NAA) on root development. Cutting of initial primary root of 4 months old *in vitro* grown plants was done (leaving about 0.5 – 1 cm root) as practiced in coconut embryo culture (Weerakoon *et al.*, 2002). Root development in 3 treatments *viz.*, dipping shoots in IAA solution (10<sup>-4</sup> M) for 3 days followed by transfer to normal embryo culture media (Fernando & Gamage, 1995); culturing in 10 µM BAP and 10 µM kinetin added media for 1 month followed by transfer to 200 µM NAA added media; culturing in 10 µM BAP, 10 µM kinetin and 200 µM NAA added media for 1 month followed by transfer to normal embryo culture media, were compared with the control (culturing in normal embryo culture media). Three replicates with 15 plants in one replicate were used for each treatment. Development of new primary roots and plant growth was recorded.

### Data analysis

Continuous data were analysed using GLM procedure and count data were analysed using CATMOD procedure in SAS statistical package (SAS Institute Inc., 1999).

## RESULTS AND DISCUSSION

### Effect of genotype on *in vitro* embryo germination

A significant difference in *in vitro* embryo germination was observed among cultivars used in this experiment (Table 1). The highest percentage of *in vitro* embryo germination was observed in the cultivar San Ramon Tall (77.48 %) and the lowest was observed in the cultivar King Coconut (52.5 %). Therefore, the effect of genotype can be considered as a factor contributing to variation of *in vitro* embryo germination of exotic varieties. Karun *et al.* (2002) also reported significant effect of the genotype and interaction between media

composition and the genotype when four coconut cultivars (West Coast Tall, Laccadive Ordinary, Chowghat Orange Dwarf, and Malayan Yellow Dwarf) were cultured in embryo culture media used in the Philippines, India and France. Parallel studies conducted in Sri Lanka revealed significant difference of *in vitro* embryo germination between the cultivar Sri Lanka Tall (SLT) and PGD in modified Y<sub>3</sub> medium (Weerakoon *et al.*, 2002). In the present study cultivars which were not tested previously were selected. SNRT, which is a tall cultivar and SLT performed better than SLRD, RTB and PGD, which are dwarf and intermediate cultivars. Composition of the modified Y<sub>3</sub> medium used in the present study was very similar to the media (Philippines, UPLB) used in the study by Karun *et al.* (2002). Cueto *et al.* (2012) confirmed the effects of genotype, age of nut, disinfection and storage procedures, screenhouse and tissue culture facilities on the establishment of embryos in culture and survival of embryo cultured seedlings during germplasm exchange programmes. Therefore, optimisation of culture media composition and culture conditions is required for different varieties to improve *in vitro* embryo germination. In addition, during germplasm exchange programmes more embryos can be collected and cultured from low germinating cultivars to get the desired number of plants from those cultivars.

**Table 1:** *In vitro* embryo germination of different cultivars

Varieties	Number of embryos cultured	Number of embryos germinated	Percentage of embryo germination
SNRT	85	66	77.48 ± 15.2 <sup>a</sup>
SLRD	86	57	67.28 ± 23.8 <sup>c</sup>
RTB	80	42	52.5 ± 6.5 <sup>d</sup>
PGD	86	62	71.85 ± 8.1 <sup>b</sup>
CV %			15.90

Mean values followed by the same letter are not significantly different at 5 % significant level

**Table 2:** Percentage of *in vitro* embryo germination of SLRD in different media

Medium	Number of embryos cultured	Number of embryos germinated	Percentage of embryo germination
Liquid 75 g/L sucrose	90	51	56.66 ± 10.6 <sup>b</sup>
Liquid 60 g/L sucrose	86	52	60.46 ± 7.5 <sup>b</sup>
Solid 75 g/L sucrose	84	77	91.66 ± 5.2 <sup>a</sup>
Solid 60 g/L sucrose	90	83	92.22 ± 5.0 <sup>a</sup>
CV %			25.7

Mean values followed by the same letter are not significantly different at 5 % significant level

### Effect of germination medium on *in vitro* embryo germination of SLRD

A significant difference in *in vitro* embryo germination in liquid and solid medium was observed. A high *in vitro* embryo germination percentage was observed in solid medium compared to that in liquid medium (Table 2). However, the sucrose levels tested had no significant effect on *in vitro* embryo germination.

Shoot height in monthly intervals up to three months indicated a significant variation in shoot growth in different culture media (Table 3). A higher average shoot

height was observed in solid medium compared to that in liquid medium. Moreover, 60 g/L sucrose content in the medium favoured shoot growth by achieving the highest shoot height. Further, a better root growth was also observed in solid medium (data not statistically significant). Rillo and Cueto (2003) reported that at least one passage in solid medium and reduction of sugar during the growth and development phase improve *in vitro* growth in terms of earlier leaf and root formation, and higher percentage of cultures with simultaneous shoot and root formation. Karun *et al.* (2002) also reported hyperhydricity of embryos when germinated in liquid medium.

**Table 3:** Average shoot height of SLRD plants with time in different culture media

Medium	Average shoot height after 1 month (mm)	Average shoot height after 2 months (mm)	Average shoot height after 3 months (mm)
Liquid 75 g/L sucrose	5.52 ± 4.2 <sup>c</sup>	15.0 ± 13.3 <sup>d</sup>	21.18 ± 19.9 <sup>c</sup>
Liquid 60 g/L sucrose	5.52 ± 4.4 <sup>c</sup>	15.15 ± 15.7 <sup>c</sup>	19.65 ± 22.7 <sup>d</sup>
Solid 75 g/L sucrose	6.64 ± 4.2 <sup>b</sup>	27.8 ± 8.3 <sup>b</sup>	41.04 ± 14.3 <sup>b</sup>
Solid 60 g/L sucrose	7.5 ± 3.9 <sup>a</sup>	29.26 ± 9.8 <sup>a</sup>	48.26 ± 29.3 <sup>a</sup>
CV %	15.27	35.74	44.0

Mean values followed by the same letter in a column are not significantly different at 5 % significant level

Improvement in the percentage of *in vitro* germination may be due to better aeration of embryos in solid medium as embryos were placed upward up to about 2/3 of its height buried in the medium. The effect of embryo placement in culture medium on *in vitro* germination has been studied by Areza *et al.* (1995) and a higher embryo germination was observed in solid medium compared to liquid medium. Further, Areza *et al.* (1995) indicated variations in germination percentage with embryo placement such as upward embryo placement in solid medium (93 %); downward embryo placement in solid medium (56 %); horizontal embryo placement in solid medium (88 %) and submerged in liquid medium (66 %).

### Effect of embryo maturity on *in vitro* germination of PGD

The highest percentage of *in vitro* germination was observed in embryos of the 12 month old bunch (97.67 %), while the lowest percentage of germination was observed in the 10 month old bunch (52.17 %). These results clearly showed that *in vitro* embryo germination was affected by maturity of the nuts (Table 4).

A similar trend was observed in a previous experiment using a 9 month old bunch and 10 month old bunch from the cultivar Sri Lanka Tall (Weerakoon, 1999). An embryo germination percentage of 91.3 % was observed in the 10 month old bunch compared to 27.3 % in the 9 month old bunch (Weerakoon, 1999). Further, the results of an experiment conducted using embryos from 9<sup>th</sup> to 11<sup>th</sup> bunches of the cultivar Mapanget Tall

**Table 4:** *In vitro* germination of embryos of PGD at different maturity stages

Maturity stage of embryo	Number of embryos cultured	Number of embryos germinated	Percentage embryo germination
10 months	46	24	52.17 ± 21.7 <sup>c</sup>
11 months	42	35	83.33 ± 13.3 <sup>b</sup>
12 months	43	42	97.67 ± 8.0 <sup>a</sup>
CV %			29.93

Mean values followed by the same letter are not significantly different at 5 % significant level.

indicated 36.67, 73.33 and 78.33 % embryo germination in 9<sup>th</sup>, 10<sup>th</sup> and 11<sup>th</sup> bunch, respectively (Nurhaini, 2002). Therefore, during germplasm collection expeditions, nut maturity should be critically considered for successful embryo germination.

### Evaluation of hormonal influence in shoot and root development

A significantly highest number of new primary roots developed (5.2) in seedlings cultured in 10  $\mu$ M BAP, 10  $\mu$ M kinetin and 200  $\mu$ M NAA added medium compared to that in the other treatments (Figure 2). Adding any growth hormone combination had a positive effect on rooting compared to the control (Table 5). Although the shoot height was lower (272.0 mm) in the above medium the number of leaves was the highest (3.6), which is a more favourable character for successful weaning. Therefore, considering the 3 parameters (number of new primary roots, shoot height, number of leaves), the addition of 10  $\mu$ M BAP, 10  $\mu$ M kinetin and 200  $\mu$ M NAA to embryo culture medium after 4 months enhances good root growth resulting in a plant suitable for weaning.

Ashburner *et al.* (1993) reported on the stimulation of primary root elongation when 100 – 300  $\mu$ M NAA was added to the final growth medium with increased sucrose level (8 %). In this experiment the sucrose content was kept at 6 %, which is the level used in all coconut embryo protocols (Weerakoon *et al.*, 2002). A significant



**Figure 2:** New primary root induction in 10  $\mu$ M BAP, 10  $\mu$ M Kinetin and 200  $\mu$ M NAA added medium

improvement was achieved by culturing in 10  $\mu$ M BAP, 10  $\mu$ M kinetin and 200  $\mu$ M NAA added medium for the initiation of many new primary roots compared to the other treatments. In preliminary experiments when 200  $\mu$ M NAA alone was tested, retardation of shoot growth (shoot height and number of leaves) was observed and this was also evident in the study by Ashburner *et al.* (1993). Shoot retardation due to root enhancement was also observed by IAA treatment (dipping shoots in IAA solution for 3 days followed by transfer to normal embryo culture media). This effect was compensated by adding 10  $\mu$ M BAP, 10  $\mu$ M kinetin at the same time of 200  $\mu$ M NAA addition.

**Table 5:** Root and shoot development of SLRD plants in different rooting treatments

Medium	Average new primary roots after 3 months	Average shoot height after 3 months (mm)	Average leaf number after 3 months
IAA	2.5 $\pm$ 0.7 <sup>b</sup>	242.0 $\pm$ 20.3 <sup>c</sup>	2.4 $\pm$ 0.93 <sup>b</sup>
BAP + kinetin	2.2 $\pm$ 0.3 <sup>c</sup>	313.3 $\pm$ 16.2 <sup>a</sup>	2.8 $\pm$ 0.85 <sup>b</sup>
BAP + kinetin + NAA	5.2 $\pm$ 0.9 <sup>a</sup>	272.0 $\pm$ 19.2 <sup>b</sup>	3.6 $\pm$ 0.98 <sup>a</sup>
Y <sub>3</sub>	0.5 $\pm$ 0.7 <sup>d</sup>	321.8 $\pm$ 19.8 <sup>a</sup>	2.8 $\pm$ 0.77 <sup>b</sup>
CV %	34.9	33.5	27.1

Mean values followed by the same letter in a column are not significantly different at 5 % significant level.

### CONCLUSION

This study indicated that factors such as genotype, embryo maturity and culture medium affected *in vitro* germination of coconut embryos. Therefore, these factors have to be considered during embryo collection and culture. During embryo collection for varieties, which

show low *in vitro* germination, more embryos have to be collected. Higher *in vitro* embryo germination can be achieved by culturing embryos from the 12 month old bunch. Embryo germination percentages could be further improved by culturing initially in solid media than in liquid media. By adding growth hormones plant growth and vigour could be improved with a significant increase in the number of primary roots and the number of leaves.

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