Plant Cell Tiss Organ Cult (2009) 99:73–81
DOI 10.1007/s11240-009-9577-z

ORIGINAL PAPER

# Effect of plant growth regulators on ovary culture of coconut (*Cocos nucifera* L.)

**P. I. P. Perera · V. R. M. Vidhanaarachchi · T. R. Gunathilake · D. M. D. Yakandawala · V. Hocher · J. L. Verdeil · L. K. Weerakoon**

Received: 21 September 2008 / Accepted: 23 July 2009 / Published online: 8 August 2009


**Abstract** Coconut is a cross pollinating palm, propagated only by seeds. Tissue culture is the only vegetative propagation method available for coconut. Consistent callogenesis was obtained by culturing unfertilised ovaries at -4 stage in CRI 72 medium containing 100 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1% activated charcoal. Callusing was improved by application of 9 μM thidiazuron (TDZ). Embryogenic calli were subcultured onto somatic embryogenesis induction medium containing 66 μM 2,4-D. Stunted growth was observed in the somatic embryos after subculture onto CRI 72 medium containing abscisic acid (ABA). Maturation of somatic embryos could be achieved in $Y_3$ medium without growth regulators. Conversion of somatic embryos was induced by adding gibberellic acid ($GA_3$) to conversion medium containing 5 μM 6-benzyladenine (BA) while 2-isopentyl adenine (2iP) increased the frequency of plant regeneration. A total of 83 plantlets was produced from 32 cultured ovaries.

**Keywords** Callogenesis · Regeneration · Somatic embryogenesis · Palm · Arecaceae

**Abbreviations**

| | |
|---|---|
| ABA | Abscisic acid |
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| BA | 6-Benzyladenine |
| 2iP | 2-Isopentyl adenine |
| $GA_3$ | Gibberellic acid |
| TDZ | Thidiazuron |

P. I. P. Perera (✉) · V. R. M. Vidhanaarachchi · T. R. Gunathilake · L. K. Weerakoon
Tissue Culture Division, Coconut Research Institute, Lunuwila 61150, Sri Lanka
e-mail: prasanthi1970@yahoo.com

D. M. D. Yakandawala
Department of Botany, University of Peradeniya, Peradeniya, Sri Lanka

V. Hocher
Institute for Research and Development (IRD), UMR 1098 BEPC, IRD, 911 Av. Agropolis, BP 64501, 34394 Montpellier Cedex 1, France

J. L. Verdeil
CIRAD, TA40/02 Avenue Agropolis, 34398 Montpellier Cedex 5, France

P. I. P. Perera · D. M. D. Yakandawala
Postgraduate Institute of Science, University of Peradeniya, Peradeniya, Sri Lanka

## Introduction

Grown in about 90 countries, the coconut palm (*Cocos nucifera* L.) is an important perennial tropical plantation crop. It occurs in the palm family (Arecaceae) and is the sole species of the genus *Cocos* within the subfamily Arecoideae which also contains other economically important species including date palm and oil palm (Dransfield and Uhl 1986). All forms of coconut known to date are diploid ($2n = 2x = 32$).

Coconut is a cross pollinating crop propagated only by seed. Consequently, phenotypic variation within plantations hinders agronomic practices. Homogenous planting materials for the coconut industry through vegetative propagation would alleviate the problem. Tissue culture has been the only possible clonal propagation method available for coconut. Previous studies on somatic embryogenesis in coconut have been conducted using different explant types including immature inflorescences (Branton and Blake 1983; Verdeil et al. 1994) and tender leaves (Pannetier and Buffard-Morel 1982; Buffard-Morel et al. 1988); the response of these somatic tissues to in vitro culture has been low and inconsistent. Even though zygotic tissues such as immature embryos (Karunaratne and Periyapperuma 1989; Fernando and Gamage 2000) and plumules (Hornung 1995; Chan et al. 1998; Fernando et al. 2003) showed better response, these are not ideal for clonal propagation since they produce clones with unknown performance due to heterogeneity among seedlings after cross pollination in coconut.

The ovary is a potential tissue for somatic embryogenesis due to the juvenilizing influence of nearby meiotic tissues (Bonga 1982). Plant regeneration has been achieved via ovary culture of several crops such as onion (*Allium cepa*; Bohanec et al. 1995; Luthar and Bohanec 1999), sweetpotato (*Ipomoea batatas*; Ruth et al. 1993), lily (*Lilium* spp.; Van Tuyl et al. 1991), tulip (*Tulipa generiana*; Van Creij et al. 2000), maize (*Zea mays*; Tang et al. 2006) and sugar beet (*Beta vulgaris*; Gurel et al. 2000). In vitro culture of unfertilized ovaries of coconut has resulted in callus and adventitious root formation; however, no incidence of somatic embryogenesis has been observed (Griffis and Litz 1997). Suitable culture conditions have been developed for consistent callogenesis from unfertilized ovaries of coconut (Perera et al. 2007). This report in combination with a protocol developed for callus multiplication (Perez-Nunez et al. 2006) suggest the possibility of mass production of homogenous planting materials of improved coconut varieties. Even though consistent callusing was reported, the callogenesis frequency (30%) and the plant regeneration efficiency were low (Perera et al. 2007).

In the present study attempts were made to enhance callogenesis and plant regeneration efficiency by incorporating different growth regulators into the culture medium. Thidiazuron has been found to be a potent cytokinin for promoting callus formation in woody plants (Huetteman and Preece 1993). ABA has been used to induce somatic embryogenesis in plumule culture of coconut (Fernando and Gamage 2000). Perera et al. (2007) induced somatic embryogenesis by subculturing ovary-derived calli of coconut onto CRI 72 medium containing ABA, with low plant regeneration efficiency. Regeneration efficiency has been improved in monocots and woody plant species by supplementing 2iP into the culture medium in combination with other auxins or cytokinins (Levi and Sink 1991; Fraser and Harvey 1986). Incorporation of $GA_3$ has also improved plant regeneration in different crop species. Thus in the present study TDZ, ABA, 2iP and $GA_3$ were tested for somatic embryogenesis and plant regeneration in coconut ovary culture.

## Materials and methods

### Plant material

Unfertilized ovaries excised from immature female flowers of adult coconut palms (‘Sri Lanka Tall’) were used as explants. Ovaries were obtained from inflorescences of -4 stage that has an average length of 48 cm (Fig. 1a). The female flowers attached to the basal part of each rachilla (Fig. 1b, c) were collected and the two male flowers flanking each female flower (Fig. 1d) were removed. Then the female flowers were disinfected with 2% clorox for 12 min followed by four rinses with sterile water. The excision of ovaries from female flowers was carried out in a laminar flow cabinet. The perianth segments of each female flower were removed and the ovaries (about 1–3 mm in diameter; Fig. 1e) were dissected and cultured in Petri plates containing culture media.

### Preparation of culture media

Analar-grade chemicals were used for preparation of all culture media. All growth regulators were obtained from Sigma Chemical Company (USA). The pH of all media was adjusted to 5.8 before adding 0.1% (w/v) activated charcoal (BDH acid-washed). Agar was added at 0.55% (w/v). Culture media were sterilized by autoclaving at 121°C and 1.1 kg $cm^{-2}$ pressure for 15 min. After sterilization, culture media were dispensed into Petri plates (25 ml per each petri plate; 100 × 10 mm; Sterillin) in a laminar flow cabinet.

### Culture conditions for callogenesis

CRI 72 medium (Karunaratne and Periyapperuma 1989) supplemented with 100 μM 2,4-D and 4% (w/v) sucrose (Perera et al. 2007) was used for callus induction. Different levels of TDZ (9 and 18 μM/l) in callus induction medium were compared with CRI 72 medium devoid of TDZ. The ear-like structures (Perez-Nunez et al. 2006) that developed in the basal callus induction medium were subcultured onto the same medium for multiplication. Number of calli that developed from a single original callus was recorded after 8 weeks. All cultures were maintained in the dark at 28°C for 10 weeks without subculture. The percentage of callus production in different treatments was recorded.

### Somatic embryogenesis and plant regeneration

#### *Effect of 2iP and ABA*

Well-developed calli were transferred to CRI 72 medium containing 66 μM 2,4-D for 4 weeks. Four treatments

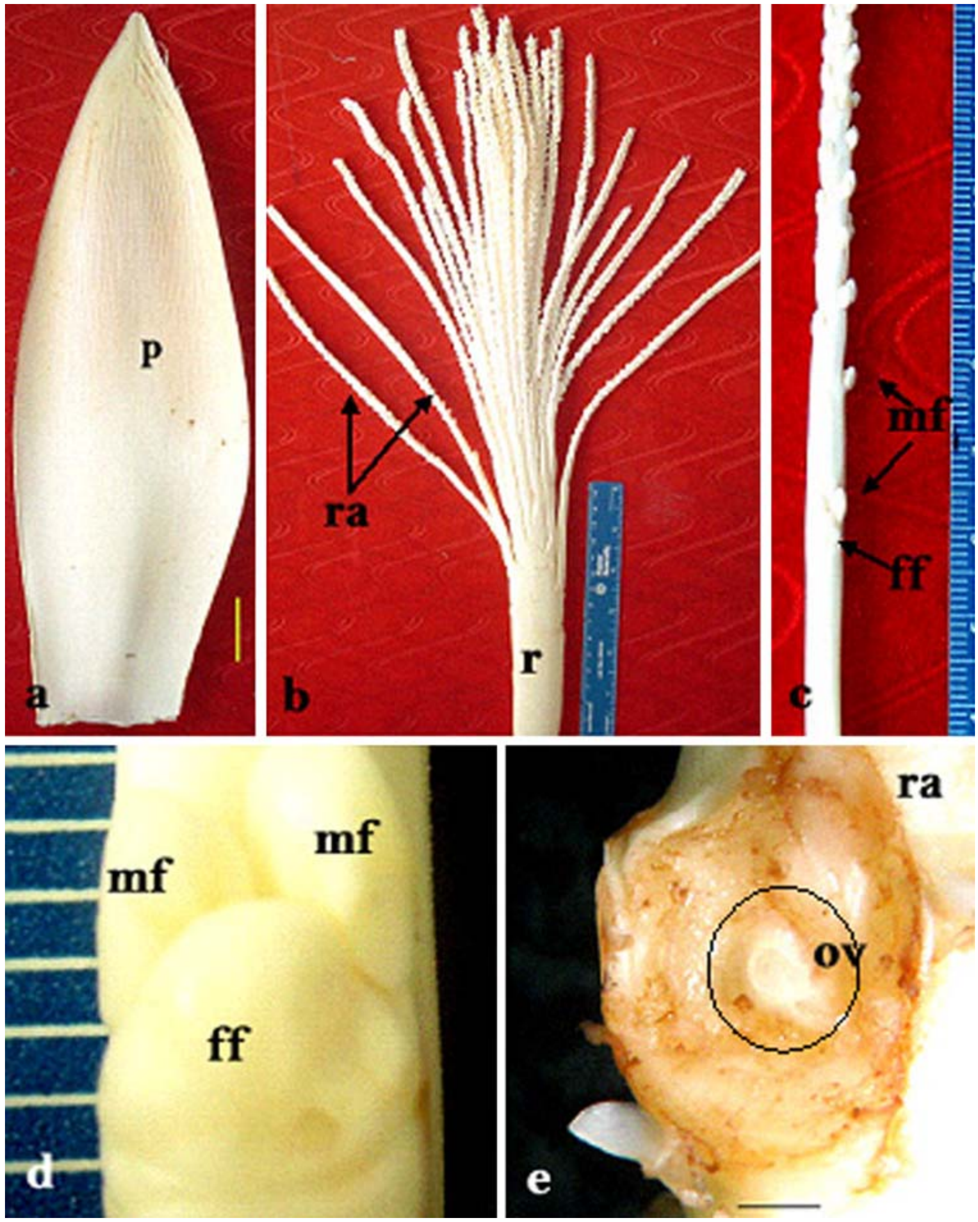


**Fig. 1** Isolation of ovaries from coconut inflorescence **a** A spadix at -4 stage (Bar = 3 cm). Note that the inflorescence is fully covered by the prophyll *(p)* **b** Inflorescence after removal of both prophyll and peduncular bract *(r-rachis; ra- rachilla)* **c** A single rachilla containing male *(mf)* and female flowers *(ff)* **d** A female flower flanked by two male flowers *(length between two bars* = 1 mm) **e** Ovary *(ov)* after removal of all perianth segments *(Bar = 1* mm)

($T_1$–$T_4$ as listed below) were applied with either filter sterilised ABA or 2iP for induction of somatic embryogenesis and plant regeneration in callus initiated from ovary explants with the aim of enhancing the plant regeneration efficiency.

$T_1$: ABA + CRI 72 medium → maturation medium ($Y_3$ without growth regulators) → conversion medium ($Y_3$ + 2iP + 2,4-D + BA)
$T_2$: ABA + CRI 72 medium → maturation medium ($Y_3$ without growth regulators) → conversion medium ($Y_3$ + 2,4-D + BA)
$T_3$: Maturation medium ($Y_3$ without growth regulators) → conversion medium ($Y_3$ + 2iP + 2,4-D + BA)
$T_4$: Maturation medium ($Y_3$ without growth regulators) → conversion medium ($Y_3$ + 2,4-D + BA)

Treatments $T_1$ and $T_2$ employed a series of three media (embryo induction, embryo maturation and embryo conversion) after the calli had developed on low-2,4-D medium. The embryo induction phase on CRI 72 medium + 5 μM ABA was for 4 weeks, followed by maturation phase (CRI 72 medium without growth regulators) for 4 weeks, then embryo conversion phase [modified Eeuwens $Y_3$ medium supplemented with 5 μM 6-benzyladenine (BA) and 0.1 μM 2,4-D]. The effect of 2iP (in the $T_1$ conversion medium) was compared with no 2iP in $T_2$ conversion medium. Embryogenic structures were then subcultured to induce somatic embryogenesis. Effect of 2iP (in the conversion medium) was compared with $T_2$ that was devoid of 2iP. The embryo induction phase was eliminated in treatments $T_3$ and $T_4$ and the same conversion medium treatments (2iP) were compared. The cultures were maintained in the dark at 28°C until the somatic embryos converted. Then the cultures were maintained under a 16 h photoperiod (PAR; 25 µmol $m^{-2}$ $s^{-1}$) at 28°C and subcultured at monthly intervals up to the acclimatisation stage.

The experiment was repeated two times with a minimum of eight replicates (eight calli). The number of shoots produced in each treatment was recorded. However, little shoot formation was observed in all four treatments, even after subculturing the embryogenic structures onto fresh conversion medium four times. All of the embryogenic structures were then transferred to a different conversion medium (without 2iP) supplemented with 0.35 µM gibberellic acid ($GA_3$) (filter-sterilized) to induce shoot formation. When plantlets exhibited a well-developed root system, they were subcultured onto liquid conversion medium.

*Effect of $GA_3$*

Based on the observations of the previous study, an experiment was conducted to identify the effect of $GA_3$ concentration (0 -control, 0.2, 0.35 and 0.45 µM) for shoot development. The experiment was repeated two times. In these experiments, calli were first subcultured onto CRI 72 medium supplemented with 66 µM 2,4-D followed by maturation medium ($Y_3$ medium devoid of any growth regulators) and conversion medium ($Y_3$ medium + 5 µM BA and 0.1 µM 2,4-D, either with or without $GA_3$). The cultures were incubated under the conditions described above. Based on the results of this experiment another experiment was conducted to test additional levels of $GA_3$ (0.45 as the control, 0.75, 1.0, 1.5, 2.0 µM) to identify the optimum concentration of $GA_3$ on plant regeneration.

*Combined effect of 2iP and $GA_3$*

Based on the above results, a combination of 2iP and $GA_3$ was tested for plant regeneration. The embryogenic structures that developed on maturation medium were subcultured onto three different conversion media ($Y_3$ medium with; 5 µM BA and 0.1 µM 2,4-D; 5 µM BA and 0.1 µM 2,4-D + 0.35 µM $GA_3$; 5 µM BA and 0.1 µM 2,4-D + 0.35 µM $GA_3$ + 5 µM 2iP). The cultures were incubated under the conditions described above.

Statistical analysis

After 10 weeks of culture initiation, number of calli produced (from cultured ovaries) in different treatments was recorded. Number of shoots produced in each of the regeneration experiments was also recorded. The data were analysed using SAS statistical package (SAS Institute 1999). Chi-square or Maximum likelihood analysis of variance was conducted using the Proc CatMod procedures of PC-SAS. Treatment means were compared using SE, 95% confidence intervals or orthogonal contrast coefficients, where appropriate (Compton 1994).

## Results and discussion

Effect of TDZ on callogenesis

After 10 weeks of culture, off white calli consisting of translucent masses of globules (Fig. 2a) could be observed in all treatments. The callus induction medium supplemented with 9 µM TDZ gave rise to the greatest callusing frequency (76.4%) indicating a positive effect of TDZ on callusing (Table 1). Further increase of the TDZ up to18 µM reduced the callogenesis frequency (67%). However, the results were not statistically significant. Induction of somatic embryogenesis in ovary and ovule cultures of *Spathiphyllum* was possible by using a medium enriched with TDZ (0.25–1 µM). However, at higher concentrations, TDZ induced diploid parthenogenesis (Eeckhaut et al. 2001). TDZ was found to be the most effective cytokinin for callus induction in carob (*Ceratonia siliqua*) anther culture and high frequencies of callogenesis have been observed with 4 mg/l (18 µM) TDZ in combination with 2,4-D (Custodio et al. 2005). The capacity of TDZ to stimulate morphogenesis in anther-derived calli of apricot (*Prunus armeniaca*) was reported by Peixe et al. (2004).

Callus multiplication

Multiplication of ovary-derived callus was possible under the culture conditions employed 6 weeks after callus inoculation. The maximum rate of multiplication achieved was 25. However, the multiplication rate depended on the quality (embryogenic or non-embryogenic) and the size (number of globules per callus) of the original callus. Many embryogenic systems can be perpetuated *via* repetitive embryogenesis that makes them potentially attractive for mass production of clonal plants. Theoretically, a culture initiated from a single explant can produce an unlimited number of embryos (Parrott et al. 1991). Recently, Perez-Nunez et al. (2006) reported a highly efficient system of plant regeneration *via* somatic embryogenesis in plumule explants of coconut in which about 100,000 somatic embryos could be produced from a single plumule explant. This has been achieved by multiplication of embryogenic

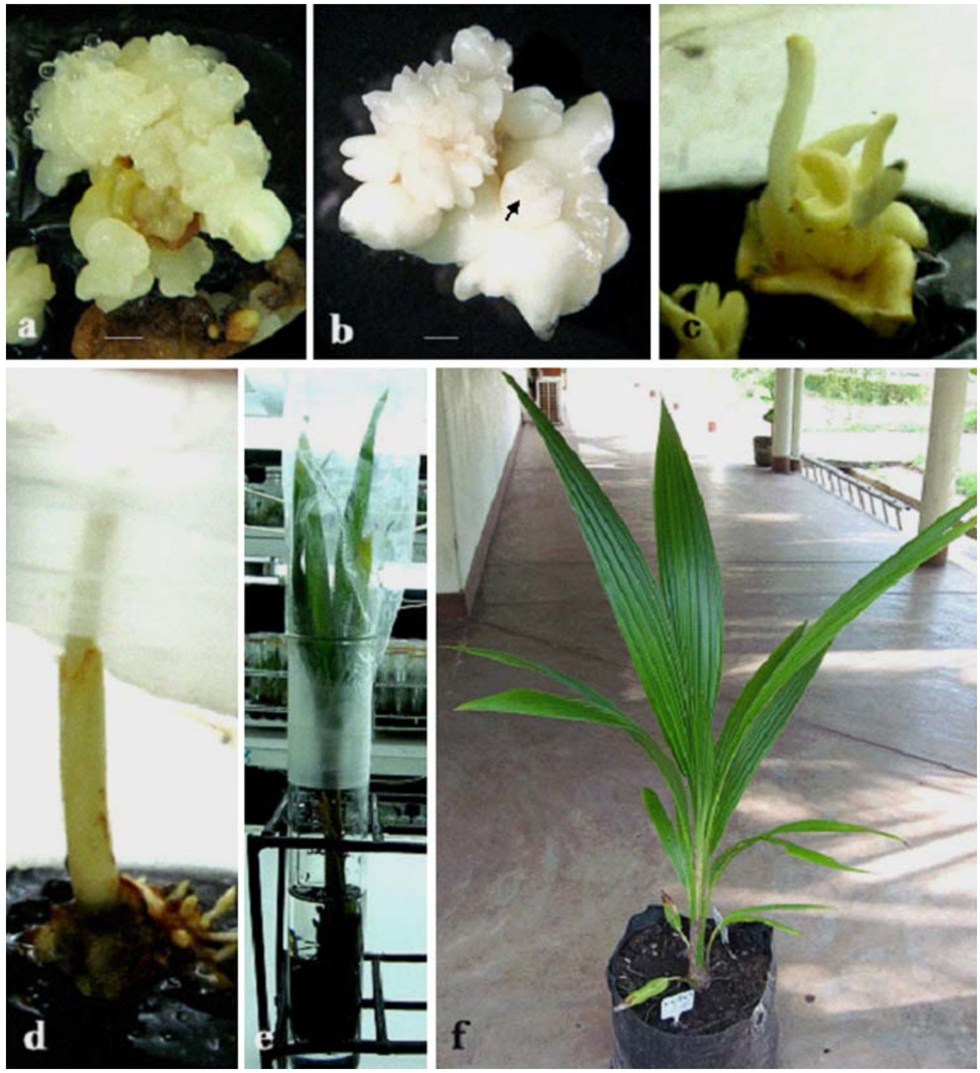


**Fig. 2** Plant regeneration *via* ovary culture of coconut (*Cocos nucifera* L.). **a** Ovary-derived callus (*Bar* = 1 mm) produced in callogenesis induction medium. **b** Somatic embryos obtained after subculturing the calli onto somatic embryo maturation medium devoid of growth regulators (*Bar* = 1.1 mm). Note the converted somatic embryo indicated by an *arrow*. **c** Early stage of shoot development of a converted somatic embryo **d** Shoot and root development of the plantlet in conversion medium containing $GA_3$ that was maintained in the dark. **e** Well developed complete plantlet in liquid conversion medium containing $GA_3$. **f** Ovary-derived coconut plant transferred to the soil

**Table 1** Mean percent callus production from coconut ovaries cultured on CRI 72 medium supplemented with different concentrations of TDZ

| Concentration of TDZ (μM) | No. of ovaries cultured | Percentage callus production |
|---|---|---|
| 0 (control) | 38 | 55 |
| 9 | 46 | 76 |
| 18 | 39 | 67 |
| Significance | | NS |

callus and secondary embryogenesis. However, the use of plumules from zygotic embryos obtained by cross pollination will preclude the application of this protocol for cloning palms with known agronomic traits (Perez-Nunez et al. 2006). By applying the above approach to unfertilized ovary explants, it was possible to develop an efficient protocol to clone palms with known agronomic performance. The present study revealed that callus multiplication of unfertilized ovary explants can be used to develop a micropropagation protocol for mass propagation of coconut, a growing need of the industry.

## Plant regeneration *via* somatic embryogenesis

The calli that developed on callus induction medium consisted of off white translucent masses of globules (Fig. 2a). When they were transferred onto low 2,4-D medium, the translucent appearance of the globules gradually changed to opaque (Fig. 2b). Hornung and Verdeil (1999) also observed that coconut callus grown on media with gradual reduction of auxin eventually produced nodular somatic structures that subsequently developed into pro-embryos. According to Verdeil et al. (1994), somatic embryogenesis in coconut could be induced by an increase in the 2,4-D level followed by a decrease. The somatic embryos obtained from ovary derived-calli were identified based on morphological features including creamy colour and elongated shape (Fig. 2b).

*Effect of 2iP and ABA*

In all treatments tested, poor shoot formation was observed even after repeated subculture of somatic embryos onto the conversion medium. $GA_3$ has been used at a concentration of 0.35 µM to enhance germination of zygotic embryos of coconut (Weerakoon et al. 2002). Thus all the embryogenic structures (obtained from above experiment) were subcultured onto conversion medium supplemented with 0.35 µM $GA_3$, after four subcultures onto the above mentioned conversion media. Shoot formation was observed in the presence of $GA_3$ and the number of shoots increased with continuous exposure to $GA_3$ (Table 2).

Shoot regeneration was obtained with all four treatments tested ($T_1$, $T_2$, $T_3$ and $T_4$) with a clearly positive effect of 2iP ($T_1$ and $T_3$) and negative effect of ABA (Table 2). The greatest percentage of shoot regeneration (85%) was achieved with $T_3$ where ABA was not used for induction of somatic embryogenesis. Based on these results, the induction of somatic embryogenesis appears to occur when calli are first transferred to the low 2,4-D containing medium. Maturation of embryos occurs in medium without growth regulators and early exposure of mature embryos to 2iP seems to enhance shoot regeneration. The results indicated that subculturing the somatic embryos directly onto the conversion medium containing 5 µM 2iP and 5 µM BA could give rise to shoots at a high frequency (85%; $P < 0.01$). Even though ABA has been used for induction and maturation of somatic embryos in coconut (Fernando and Gamage 2000; Perera et al. 2007), present results suggest that it could reduce the conversion of somatic embryos into shoots. Observations revealed that the development of the somatic embryo was stunted in the presence of ABA in culture medium and only weak plants resulted. Thus, by omitting this step, plant regeneration efficiency could be increased.

2iP has been used to improve the regeneration efficiency in several plant species. For *Asparagus officinalis* (Levi and Sink 1991) and *Hordeum vulgare* (Weigel and Hughes 1985), 1.4 or 1.5 µM 2iP induced somatic embryos in combination with an auxin. Different concentrations of 2iP (in combination with an auxin or another cytokinin) have also been used for the development of somatic embryos in *Asparagus officinalis, Allium fistulosum* × *A. cepa*, *Triticum aestivum, Zea mays* and *Saccharum officinarum* (Levi and Sink 1991; Ozias-Akins and Vasil 1982; Conger et al. 1987; Srinivasan and Vasil 1986). 2iP has also been used to induce somatic embryogenesis in woody species but with limited success. In two of the woody species, *Actinidia chinensis* (Fraser and Harvey 1986) and *Coffea canephora* (Hatanaka et al. 1991), the effective concentration of 2iP for induction of somatic embryogenesis was 24.6 and 5 µM, respectively. Plant regeneration has been achieved in some palm species such as *Elaeis guineensis* (Thomas and Rao 1985) and *Phoenix dactylifera* (Tisserat 1979, 1982) with the use of 2iP. In coconut, plantlet regeneration from cultured immature inflorescence explants has been reported on MS medium supplemented with 5 µM BA and 5 µM 2iP in the presence of 2,4-D (Branton and Blake 1983).

**Table 2** Percent shoot regeneration from ovary derived callus cultured on four culture procedures differing by presence (T1 and T3) or absence (T2 and T4) of 2iP in the conversion medium and by inclusion (T1 and T2) or exclusion (T3 and T4) of an initial culture phase on CRI 72 medium with ABA

| Culture procedure | Number of calli cultured | Percentage of shoot regeneration (no. of shoots per 100 callus)[a] |
|---|---|---|
| $T_1$ | 45 | 11 |
| $T_2$ | 27 | 7 |
| $T_3$ | 16 | 85 |
| $T_4$ | 17 | 7 |
| **Contrasts** | | **Chi square** |
| $T_1$ vs. $T_3$ | | 40.47*** |
| $T_2$ vs. $T_3$ | | 40.61*** |
| $T_3$ vs. $T_4$ | | 40.22*** |

Maximum likelihood analysis of variance was significant at 0.0001 ($G^2 = 125.96$)

[a] In all four treatments ($T_1$–$T_4$), after repeated subculture onto the relevant conversion medium, embryogenic structures were subcultured onto conversion medium containing 0.35 µM $GA_3$

*** Treatment significantly different at $P \leq 0.00$ level

*Effect of $GA_3$*

Based on the results of the previous experiment, the positive effect of $GA_3$ on embryo conversion was studied. After maturation, the somatic embryos were subcultured onto conversion medium containing different levels of $GA_3$. The result of this experiment has clearly shown that incorporation of $GA_3$ in the conversion medium improved the shoot regeneration frequency (Table 3). The most effective concentration of $GA_3$ was found to be 0.45 µM that gave rise to 40% shoot regeneration. When additional levels of $GA_3$ were tested using 0.45 µM as the control, plant regeneration efficiency was increased four fold by increasing the $GA_3$ level to 1.0 or 1.5 µM. However, further increase of $GA_3$ beyond 1.5 µM reduced shoot formation. A large difference was found for plant regeneration efficiency at the same $GA_3$ level (0.45 µM) between the above two experiments (40 and 5%). Generally, in coconut, variation among individual palms results in considerable variation among experiments. Different developmental stages of plant regeneration from cultured ovaries of

**Table 3** Mean percent shoot regeneration from coconut ovary derived calli cultured on conversion medium supplemented with different concentrations of $GA_3$

| Concentration of $GA_3$ (μM) | Shoot regeneration percentage[a] (no. of shoots per 100 callus) |
|---|---|
| 0 (C) | 5 |
| 0.2 ($G_1$) | 15 |
| 0.35 ($G_2$) | 10 |
| 0.45 ($G_3$) | 40 |
| Contrasts | Chi square |
| C vs. ($G_3$) | 4.35* |
| $G_1$vs. $G_3$ | 3.7* |
| $G_2$ vs. $G_3$ | 3.7* |

Maximum likelihood analysis was significant at 0.05 ($G^2 = 9.06$)

[a] $n = 20$

* Treatment significantly different at $P \leq 0.05$ level

**Table 4** Mean percent shoot regeneration from coconut ovary derived calli cultured on Y3 conversion media with or without $GA_3$ and 2iP

| Composition of the conversion medium | Number of calli cultured | No. of shoots per 100 callus |
|---|---|---|
| Y3 (M1) | 22 | 17 |
| Y3 + 5 μM 2iP + 0.35 μM $GA_3$ (M2) | 26 | 0 |
| Y3 + 0.35 μM $GA_3$ (M3) | 22 | 70 |
| Contrast | | Chi-square |
| M1 vs. M3 | | 8.32** |

Maximum likelihood analysis of variance was significant for treatments $G^2 = 8.86$ $P \leq 0.05$

** Treatment significantly different at $P \leq 0.05$ level

coconut are shown in Fig. 2c, d a well developed complete plantlet in the liquid conversion medium is shown in Fig. 2e.

Incorporation of $GA_3$ at a concentration of 2.9 μM onto the somatic embryo conversion medium has enhanced plant regeneration in herbaceous monocots such as *Aspargus cooperi, A. officinalis, Eleusine coracana, Hordeum vulgare* and *Zea mays* (Ghosh and Sen 1991; Kunitake and Mii 1990; Sivadas et al. 1990; Kott and Kasha 1984; Radojevic 1985) whereas 0.3 μM $GA_3$ was effective for *Secale ancestrale, S. kuprianovii, S. segetale* and *S. vavilovii* (Rybczynski and Zdunczy 1986). Gmitter and Moore (1986) indicated that $GA_3$-supplemented medium was sufficient to stimulate conversion of somatic embryos derived from calli of *Citrus* ovules. Kochba et al. (1974) studied the effect of various protocols on conversion of ovule-derived somatic embryos in *Citrus* and found that $GA_3$ in combination with adenine sulphate stimulated root initiation and development. Spiegel-Roy and Vardi (1984) also reported that $GA_3$-supplemented medium stimulated conversion of *Citrus* somatic embryos. $GA_3$ has been used at concentrations as high as 10 μM [together with indoleacetic acid (IAA) or zeatin] for plant regeneration of *Citrus* spp. (Hidaka and Kajiura 1988). For oak (*Quercus robur*), 2.9 μM $GA_3$ has been used in combination with BA (Chalupa 1990).

### *Combined effect of $GA_3$ and 2iP*

Combinations of $GA_3$ and 2iP were studied to optimize plant regeneration frequency. However, combinations of the two growth regulators had a negative impact on shoot development (Table 4). Contrary to this observation, the use of a combination of $GA_3$ and 2iP in the somatic embryo development medium at concentrations of 2.9 and 4.9 μM gave rise to shoots in *Secale cereale* (Lu et al. 1984).

In the present study a total of 83 plantlets was produced from 32 cultured ovaries. However, most of the plantlets had slow growth rate and the majority was lost due to fungal contamination that occurred as a result of the poor laboratory conditions. Only three plantlets survived and were transferred to soil (Fig. 2f).

## Conclusions

Unfertilized ovaries are a promising source of explants for clonal propagation of coconut. Callus initiation and multiplication could be enhanced by incorporating TDZ into the callus induction medium. This observation is encouraging as it could lead to an efficient micropropagation protocol. Somatic embryogenesis was induced by transferring callus onto a low 2,4-D-containing medium. Maturation of somatic embryos could be achieved on basal medium and early exposure of somatic embryos to 2iP promoted shoot regeneration. The presence of $GA_3$ in conversion medium was critical for plant regeneration.

**Acknowledgments** We are thankful to J.D.J.S. Kularatne, Biometry Division, Coconut Research Institute, Lunuwila, Sri Lanka for his assistance in statistical analysis. Special thanks go to Mr. Kapila Prasanna, Tissue Culture Division, Coconut Research Institute, Sri Lanka, for careful attention in sample collection.

## References

Bohanec B, Marijana J, Alojz I, Branka J (1995) Studies of gynogenesis in onion (*Allium cepa* L.) induction procedures and genetic analysis of regenerents. Plant Sci 104:215–224

Bonga JM (1982) Vegetative propagation in relation to juvenility, maturity and rejuvenation. In: Bonga JM, Durzan DJ (eds)

Tissue culture in forestry. Martinus Nijhoff/Dr W Junk, The Hague, pp 387–412

Branton RL, Blake J (1983) Development of organized structures in callus derived from explants of *Cocos nucifera* L. Ann of Bot 52:673–678

Buffard-Morel J, Verdeil J-L, Pannetier C (1988) Vegetative propagation of coconut palm through somatic embryogenesis, obtention of plantlets from leaf expla. In: Durand G, Bobichon L, Florent J (eds) Proceedings of the 8th International Biotechnology Symposium, Paris, p 117

Chalupa V (1990) Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). Plant Cell Tiss Organ Cult 9: 398–401

Chan JL, Saenz L, Talavera C, Hornung R, Robert M, Oropeza C (1998) Regeneration of coconut (*Cocos nucifera* L.) from plumule explants through somatic embryogenesis. Plant Cell Rep 17:515–521

Compton ME (1994) Statistical methods suitable for the analysis of plant tissue culture data. Plant Cell Tiss Organ Cult 37:217–242

Conger BV, Novak FJ, Afza R, Erdelsky K (1987) Somatic embryogenesis from cultured leaf segments of *Zea mays*. Plant Cell Rep 6:345–347

Custodio L, Carneiro MF, Romano A (2005) Microsporogenesis and anther culture in carob tree (*Ceratonia siliqua* L.). Sci Hort 104:65–77

Dransfield J, Uhl NW (1986) An outline classification of palms. Principes 30:3–11

Eeckhaut T, Werbrouck S, Dendauw J, Bockstaele EV, Debergh P (2001) Induction of homozygous *Spathiphyllum wallisii* genotypes through gynogenesis. Plant Cell Tiss Organ Cult 67: 181–189

Fernando SC, Gamage CKA (2000) Abscisic acid induced somatic embryogenesis in immature embryo explants of coconut (Cocos *nucifera* L.). Plant Sci 151:193–198

Fernando SC, Verdeil J-L, Hocher V, Weerakoon LK, Hirimburegama K (2003) Histological analysis of plant regeneration from plumule explants of *Cocos nucifera* L. Plant Cell Tiss Organ Cult 72:281–284

Fraser LG, Harvey CF (1986) Somatic embryogenesis from anther-derived callus in two *Actinidia* species. Sci Hort 29:335–346

Ghosh B, Sen S (1991) Plant regeneration through somatic embryogenesis from spear callus culture of *Asparagus cooperi* Baker. Plant Cell Rep 9:667–670

Gmitter FG, Moore GA (1986) Plant regeneration from undeveloped ovules and embryogenic calli of *Citrus*: Embryo production, germination and plant survival. Plant Cell Tiss Organ Cult 6:139–147

Griffis JL, Litz RE (1997) Advances in the in vitro morphogenesis of several coconut (*Cocos nucifera* L.) tissues in Florida. International Cashew & Coconut Conference, Tanzania, pp 349–357

Gurel S, Gurel E, Kaya Z (2000) Double haploid plant production from unpollinated ovules of sugar beet (*Beta vulgaris* L). Plant Cell Rep 19:1155–1159

Hatanaka T, Arakawa O, Yasuda T, Uchida N, Yamaguchi T (1991) Effect of plant growth regulators on somatic embryogenesis in leaf cultures of *Coffea canephora*. Plant Cell Rep 10:179–182

Hidaka T, Kajiura I (1988) Plantlet differentiation from callus protoplast induced from citrus embryos. Sci Hort 34:85–92

Hornung R (1995) Initiation of callogenesis in coconut palm (*Cocos nucifera* L.). In: Oropeza C, Howard FW, Ashburner GR (eds) Lethal yellowing: research and practical aspects. Kluwer, Dordrecht, pp 203–215

Hornung R, Verdeil J-L (1999) Somatic embryogenesis in coconut from immature inflorescence explants. In: Oropeza C, Verdeil J-L, Ashburner GR, Cardena R, Santamaria JM (eds) Current advances in coconut biotechnology. Kluwer, Dordrecht, pp 297–308

Huetteman C, Preece J (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tiss Organ Cult 33: 105–119

Karunaratne S, Periyapperuma K (1989) Culture of immature embryos of coconut (*Cocos nucifera* L.): callus proliferation and somatic embryogenesis. Plant Sci 62:247–253

Kochba J, Button J, Spiegel-Roy P, Bornman CH, Kochba M (1974) Stimulation of rooting of *Citrus* embryoids by gibberellic acid and adenine sulphate. Ann Bot 38:795–802

Kott LS, Kasha KJ (1984) Initiation and morphological development of somatic embryoids from barley cell cultures. Can J Bot 62:1245–1249

Kunitake H, Mii M (1990) Somatic embryogenesis and plant regeneration from protoplasts of asparagus (*Asparagus officinalis* L.). Plant Cell Rep 8:706–710

Levi A, Sink KC (1991) Asparagus somatic embryogenesis: Production in suspension culture and conversion to plantlets on solidified medium as influenced by carbohydrate regime. Plant Cell Tiss Organ Cult 31:115–122

Lu C-Y, Chandler SF, Vasil IK (1984) Somatic embryogenesis and plant regeneration from cultured immature embryo of rye (*Secale cereale* L.). J Plant Physiol 115:237–244

Luthar Z, Bohanec B (1999) Induction of direct somatic embryogenesis in onion (*Allium cepa* L.) using a two step flower or ovary culture. Plant Cell Rep 18:797–802

Ozias-Akins P, Vasil IK (1982) Plant regeneration from cultured immature embryos and inflorescences of *Triticum aestivum* L. (wheat): evidence for somatic embryogenesis. Protoplasma 110:95–105

Pannetier C, Buffard-Morel J (1982) Premiers résultats concernant la production d'embryons somatiques à partir de tissus foliaires de cocotier, *Cocos nucifera* L. Oleagineux 37:349–354

Parrott WA, Merkle SA, Williams EG (1991) Somatic embryogenesis: potential for use in propagation and gene transfer systems. In: Murray DR (ed) Advanced methods in plant breeding and biotechnology. CAB International, UK, pp 158–200

Peixe A, Barroso J, Potes A, Pais MS (2004) Induction of haploid morphogenic calluses from in vitro cultured anthers of *Prunus armeniaca* cv. 'Harcot'. Plant Cell Tiss Organ Cult 77: 35–41

Perera PIP, Hocher V, Verdeil J-L, Doulbeau S, Yakandawala DMD, Weerakoon LK (2007) Unfertilized ovary: a novel explant for coconut (*Cocos nucifera* L.) somatic embryogenesis. Plant Cell Rep 26:21–28

Perez-Nunez MT, Chan JL, Saenz L, Gonzalez T, Verdeil JL, Oropeza C (2006) Improved somatic embryogenesis from coconut (*Cocos nucifera* L.) plumule explants cultured in vitro. In Vitro Cell Dev Biol-Plant 42:37–43

Radojevic L (1985) Tissue culture of maize *Zea mays* (Cudu) I. Somatic embryogenesis in the callus tissue. J Plant Physiol 119:435–441

Ruth SK, Stephen LS, John CB (1993) Ovule culture of sweet potato *(Ipomoea batatas*) and closely related species. Plant Cell Tiss Organ Cult 32:77–82

Rybczynski JJ, Zdunczy KW (1986) Somatic embryogenesis and plant regeneration in the genus *Secale* I. Somatic embryogenesis and organogenesis from cultured immature embryos of five wild species of rye. Theor Appl Genet 73:267–271

SAS Institute Inc. (1999) SAS/STAT user's guide, version 7–1. SAS Institute, Cary

Sivadas P, Kothari SL, Chandra N (1990) High frequency embryoid and plantlet formation from tissue cultures of the finger millet (*Eleusine coracana* L.). Plant Cell Rep 9:93–96

Spiegel-Roy P, Vardi A (1984) Citrus. In: Ammirato PV, Evans DR, Sharp WR, Yamada Y (eds) Handbook of plant cell culture, vol 3. Macmillan, New York, pp 355–372

Srinivasan C, Vasil IK (1986) Plant regeneration from protoplasts of sugarcane (*Saccharum officinarum* L.). J Plant Physiol 126: 41–48

Tang F, Tao Y, Zhao T, Wang G (2006) In vitro production of haploid and double haploid plants from pollinated ovaries of maize (*Zea mays*). Plant Cell Tiss Organ Cult 84:233–237

Thomas V, Rao PS (1985) In vitro propagation of oil palm (*Elaeis giineensis* Jacq. var. tenera) through somatic embryogenesis in leaf-derived callus. Current Sci 54:184–185

Tisserat B (1979) Propagation of date palm (*Phoenix dactylifera* L.) in vitro. J Exp Bot 30:1275–1283

Tisserat B (1982) Factors involved in the production of plantlets from date palm callus cultures. Euphytica 31:201–214

Van Creij MGM, Kerckhoffs DMFJ, De Bruijn SM, Vreugdenhil D, Van Tuyl JM (2000) The effect of medium composition on ovary-slice culture and ovule culture in interspecific *Tulipa gesneriana* crosses. Plant Cell Tiss Organ Cult 60:61–67

Van Tuyl JM, Van Dien MP, Van Creij MGM, Van Kleinwee TCM, Franken J, Bino RJ (1991) Application of in vitro pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. Plant Sci 74:115–126

Verdeil J-L, Huet C, Grosdemange F, Buffard-Morel J (1994) Plant regeneration from cultured immature inflorescence of coconut (*Cocos nucifera* L.): evidence for somatic embryogenesis. Plant Cell Rep 13:218–221

Weerakoon LK, Vidhanaarachchi VRM, Fernando SC, Fernando A, Gamage CKA (2002) Increasing the efficiency of embryo culture technology to promote coconut germplasm collecting and exchange in Sri Lanka. In: Engelmann F, Batugal P, Oliver J (eds) Coconut embryo in vitro culture: part II. IPGRI-APO, Malaysia, pp 27–40

Weigel RC, Hughes KW (1985) Long term regeneration by somatic embryogenesis in barley (*Hordeum vulgare* L.) tissue cultures derived from apical meristem explants. Plant Cell Tiss Organ Cult 5:151–162