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2 **Initiation of embryogenic cell suspensions of taro**  
3 **(*Colocasia esculenta* var. *esculenta*) and plant regeneration**

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8 **Abstract** Embryogenic callus was initiated by culturing  
9 in vitro taro corm slices on agar-solidified half-strength MS  
10 medium containing 2.0 mg/L 2,4-dichlorophenoxyacetic  
11 acid (2,4-D) for 20 days followed by transfer to 1.0 mg/L  
12 thidiazuron (TDZ). Callus was subsequently proliferated  
13 on solid medium containing 1.0 mg/L TDZ, 0.5 mg/L 2,4-  
14 D and 800 mg/L glutamine before transfer to liquid med-  
15 ium containing the same components but with reduced  
16 glutamine (100 mg/L). After 3 months in liquid culture on  
17 an orbital shaker, cytoplasmically dense cell aggregates  
18 began to form. Somatic embryogenesis was induced by  
19 plating suspension cells onto solid media containing  
20 reduced levels of hormones (0.1 mg/L TDZ, 0.05 mg/L  
21 2,4-D), high concentrations of sucrose (40–50 g/L) and  
22 biotin (1.0 mg/L). Embryo maturation and germination was  
23 then induced on media containing 0.05 mg/L benzylade-  
24 nine (BA) and 0.1 mg/L indole-3-acetic acid (IAA). His-  
25 tological studies of the developing embryos revealed the  
26 presence of typical shoot and root poles suggesting that  
27 these structures were true somatic embryos. The rate of  
28 somatic embryos formation was 500–3,000 per mL settled

cell volume while approximately 60% of the embryos 29  
regenerated into plants. 30

**Keywords** Somatic embryogenesis · Callus · 31  
Cell suspension · Taro · *Colocasia esculenta* var. *esculenta* 32  
33

**Introduction** 34

Taro (*Colocasia esculenta* var. *esculenta*) is an important 35  
food crop grown throughout many Pacific Island countries. 36  
In addition to contributing to sustained food security in the 37  
domestic market, it also provides a source of export earn- 38  
ings in some countries. Since taro is largely asexually 39  
propagated (Strauss et al. 1979; Ivancic 1992), there is little 40  
genetic variation within cultivars. Consequently, it is sus- 41  
ceptible to numerous pests and diseases which can place 42  
serious constraints on production (Ivancic 1992). 43

The use of conventional breeding to obtain pest and/or 44  
disease resistant taro cultivars has been hampered by 45  
numerous problems including the unavailability of resistant 46  
cultivars, sexual incompatibility between parents, and 47  
variable climatic conditions affecting pollination and fer- 48  
tilization rates (Wilson 1990). Molecular breeding is an 49  
attractive alternative strategy as a single trait can be added 50  
to an already accepted cultivar in a single step without the 51  
requirement for further breeding. An essential pre-requisite 52  
for molecular breeding, however, is the availability of 53  
suitable target tissue from which large numbers of trans- 54  
genic plants can be generated. 55

Embryogenic cells from liquid culture represent a suit- 56  
able candidate target tissue for transformation as (1) the 57  
unicellular origin of somatic embryos reduces the likeli- 58  
hood of chimerism and (2) the relatively small size of cell 59  
clumps creates a large surface for exposure to the 60

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transforming agent and more effective selection of transformants. In addition, the rapid growth of cells in liquid culture in comparison to solid media means embryogenic cell suspensions can be used as an efficient means of producing large numbers of plants with reduced space requirements and labor costs.

Adventitious shoot production from callus has been reported in *C. esculenta* var. *antiquorum* using medium containing naphthalene acetic acid (NAA) and kinetin (Abo El-Nil and Zettler 1976) and in *C. esculenta* var. *esculenta* using taro corm extract (TE) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Yam et al. 1990). Recently, we reported the development of an efficient protocol for initiating embryogenic callus from corm slices of in vitro taro plantlets (Deo et al. 2009). To enhance the utility of this system for generating transgenics and particularly as a method for mass propagation, a protocol for proliferating embryogenic callus was required. In this paper, we examined the effects of 2,4-D, TDZ, glutamine and sucrose concentrations on embryogenic callus proliferation, both on solid media and as suspension cultures, and report a protocol for initiating embryogenic taro cell suspensions from which plants can be easily regenerated.

## Materials and methods

### Source of plant material

A virus-free accession of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK (originally derived from Cook

Islands) was sourced from the Centre for Pacific Crops and Trees (CePaCT)-Secretariat of the Pacific Community (SPC), Fiji.

### Callus initiation and proliferation on solid medium

Embryogenic callus was initiated from corm slices of in vitro plantlets as previously described (Deo et al. 2009). The incubation temperature in all experiments was 25°C and unless otherwise stated all culture steps were in the dark. The culture medium for callus proliferation consisted of half-strength MS medium, 30 g/L sucrose, 7 g/L agar with a pH of 5.8 and various combinations of 2,4-D, TDZ and glutamine. Filter sterilized glutamine was added to the medium after autoclaving. Hereafter, callus proliferation medium is referred to as callus maintenance medium (CMM). Callus produced by corm slices (Deo et al. 2009) and deemed to be embryogenic by the presence of translucent globular structures was cut into equal sizes (~2 mm × 2 mm × 1 mm) and placed on CMM. For each combination of 2,4-D, TDZ and glutamine, seven to ten pieces of callus were placed on each of seven CMM plates. The precise combinations of 2,4-D, TDZ and glutamine are described in Tables 1 and 2. Cultures were checked periodically for callus growth and after 2 months without subculture, the fresh weight of each callus piece was recorded as an indicator of proliferation. Following the first 2 months on CMM, embryogenic callus was maintained by monthly subculture onto fresh CMM.

**Table 1** The effect of TDZ and 2,4-D on embryogenic callus proliferation of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK on solid medium

Plant growth regulator (mg/L)		Total number of callus pieces inoculated	Total number of callus pieces undergoing necrosis	Total number of callus pieces surviving	Mean % of callus pieces per replicate Petri dish undergoing proliferation	Mean fresh weight per callus piece (mg)
TDZ	2,4-D					
0	0	49	29	20	0 <sup>†</sup>	0 <sup>†</sup>
0	0.5	49	22	27	20.4 ± 5.3c	16.5 ± 4.4bc
0	1	49	2	47	20.4 ± 7.5c	7.9 ± 2.4d
0.5	0	49	26	23	0 <sup>†</sup>	0 <sup>†</sup>
0.5	0.5	70	1	69	85.7 ± 6.1b	14.4 ± 0.96c
0.5	1	49	1	48	95.9 ± 2.6ab	21.7 ± 2.6b
1	0	49	42	7	6.1 ± 6.1d	0.57 ± 0.57e
1	0.5	49	0	49	100 ± 0.0a	34.5 ± 2.6a
1	1	49	1	48	97.9 ± 2.1ab	23.3 ± 3.4b

Mean fresh weights are based only on those pieces which did not become necrotic

Values with means ± SEM are derived from seven replicate Petri dishes with 7–10 callus explants per replicate. Within a column, means followed by the same letters are not significantly different ( $P < 0.05$ )

<sup>†</sup> callus on these treatments turned necrotic, hence weight was not recorded

**Table 2** The effect of glutamine on embryogenic callus proliferation of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK on solid medium

Glutamine (mg/L)	Total number of callus pieces inoculated	Number of callus pieces undergoing necrosis	Number of callus pieces surviving	Mean % of callus pieces per replicate Petri dish undergoing proliferation	Mean fresh weight per callus piece (mg)
0	49	0	49	100 ± 0.0a	34.5 ± 2.6b
800	52	8	44	88.0 ± 4.9a	52.7 ± 10.1a
1,600	46	16	30	64.0 ± 6.0b	22.4 ± 6.9b
2,400	50	4	46	92.0 ± 3.7a	25.0 ± 4.7b

Mean fresh weights are based only on those pieces which did not become necrotic

Values with means ± SEM are derived from five replicate Petri dishes with 8–12 callus pieces per replicate. Within a column, means followed by the same letters are not significantly different ( $P < 0.05$ )

## 116 Initiation, maintenance and optimization of suspension 117 cultures

118 Approximately 0.5 g of embryogenic callus was placed in  
119 10 mL of liquid medium in a 100 mL Erlenmeyer flask and  
120 agitated on an orbital shaker at 90 rpm. This medium was  
121 the same as CMM except agar was omitted and the con-  
122 centration of glutamine was reduced to 100 mg/L. After  
123 7 days, 10 mL of fresh medium was added. After an  
124 additional 7 days, the cells were allowed to settle to the  
125 bottom of the flask and 10 mL of the supernatant was  
126 removed and replaced with an equal volume of fresh  
127 medium. Half the media was refreshed a second time after  
128 which the entire contents of the flask were transferred to  
129 250 mL flasks and the volume was made up to 50 mL.  
130 Henceforth, subculture was at 7-day intervals by either  
131 replacing 40 mL of old medium or dividing the cells  
132 between two flasks. When cells were of sufficient density,  
133 they were divided between flasks by allowing cells to  
134 settle, removing 30 mL of old medium, re-suspending  
135 cells, dividing the remaining 20 mL equally between flasks  
136 and adding 40 mL of fresh medium. In general, the volume  
137 of cells in 250 mL flasks was maintained as 1–2 mL settled  
138 cell volume (SCV) in 50 mL of medium. The effects of  
139 higher concentrations of glutamine (400 and 800 mg/L)  
140 and sucrose (20 g/L) on proliferation and regeneration of  
141 suspensions cells were also investigated.

## 142 Plant regeneration from suspension cells

143 Four embryogenesis media (EM) were examined for their  
144 efficacy at inducing embryogenesis from suspension cells.  
145 EM had the same components as liquid CMM except  
146 the concentrations of growth regulators were altered as  
147 follows: EM<sub>1</sub> = TDZ (1.0 mg/L) + 2,4-D (0.5 mg/L);  
148 EM<sub>2</sub> = TDZ (0.1 mg/L) + 2,4-D (0.05 mg/L); EM<sub>3</sub> =  
149 TDZ (0.01 mg/L) + 2,4-D (0.005 mg/L) and EM<sub>4</sub> =  
150 Zeatin (0.1 mg/L) + NAA (0.05 mg/L).

Suspension cells were collected 4 days after sub- 151  
culture, passed through a 500 µm stainless steel mesh 152  
filter and the filtrate was collected. The cells within the 153  
filtrate were allowed to settle in graduated 50 mL Falcon 154  
tubes and sufficient supernatant was removed to leave a 155  
settled cell volume/liquid medium ratio of approximately 156  
1:5. The cells were then resuspended and 250 µL aliquots 157  
were dispensed directly onto sterile 70 mm Whatman 158  
filter paper discs overlaid on various EMs in 159  
90 mm × 15 mm Petri dishes. After determining a suit- 160  
able EM, this media was further refined by varying 161  
sucrose concentration (30, 40 and 50 g/L) and incorpo- 162  
rating biotin (1.0 mg/L). 163

After 2 months on EM, the pro-embryogenic masses 164  
(PEMs) together with the somatic embryos were removed 165  
from the filter paper and transferred to new media for 166  
maturation and germination. Two media were examined: 167  
(1) hormone-free half-strength MS (designated RM) and 168  
(2) half-strength MS containing 0.05 mg/L BA and 0.1 mg/L 169  
IAA (designated GM). The cultures were maintained in 170  
darkness for 2–3 weeks then incubated under low light 171  
intensity (5 µmoles photons m<sup>-2</sup> s<sup>-1</sup>). After 2 weeks at 172  
low light intensity, germinating embryos were transferred 173  
to higher light (25 µmoles photons m<sup>-2</sup> s<sup>-1</sup>). 174

## 175 Histology of PEMs and somatic embryos

The pro-embryogenic masses (PEMs) and somatic embryos 176  
were fixed in formaldehyde: alcohol: acetic acid (FAA) 177  
(1:1:8 v/v) for 4 days, dehydrated in a xylene and ethanol 178  
series, then infiltrated and embedded with paraplast and 179  
wax, respectively. Thin sections (6 µm) were cut using a 180  
rotary microtome. The sections were heat fixed to 3-ami- 181  
nopropyltriethoxysilane (APES)-coated glass slides, de- 182  
waxed and stained with either Ehrlich's HX and Eosin or 183  
Safranin O-Fast Green then viewed using a compound 184  
microscope (Olympus BX41). 185

## 186 Statistical analysis

187 Data were analyzed by analysis of variance (ANOVA)  
 188 using a 95% confidence interval. Where  $P < 0.05$ , signif-  
 189 icant differences between individual treatment means were  
 190 determined using Fisher's Least Significant Difference  
 191 (LSD) test. All data were analyzed by SPSS for Windows,  
 192 version 11.

193 **Results**

## 194 Callus proliferation and maintenance on solid medium

195 To investigate the parameters affecting proliferation,  
 196 embryogenic callus was removed from the original  
 197 explants and placed on half-strength MS medium con-  
 198 taining various concentrations of 2,4-D and TDZ (Table 1).  
 199 In general, the proliferation of callus increased with  
 200 increasing hormone concentration up to a TDZ and 2,4-D  
 201 combination of 1.0 and 0.5 mg/L, respectively. This was  
 202 both in terms of the percentage of callus pieces which  
 203 proliferated and the average fresh weight. Increasing the  
 204 2,4-D concentration over 0.5 mg/L resulted in a decrease in  
 205 callus proliferation. Overall, 2,4-D appeared to be more  
 206 important as in its absence there was little or no callus  
 207 proliferation even at high TDZ levels.

208 Although a combination of 1.0 mg/L TDZ and 0.5 mg/L  
 209 2,4-D was effective in inducing callus proliferation, much  
 210 of the callus became watery and non-regenerable within  
 211 3–4 weeks. In an attempt to prevent this, the effect of glu-  
 212 tamine was examined (Table 2). Although glutamine did  
 213 not have a significant effect on the percentage of callus  
 214 pieces proliferating, the use of 800 mg/L glutamine resulted  
 215 in a significant increase in the mean fresh weight per callus  
 216 pieces. Further, the callus remained firm (did not become  
 217 watery) for over a month and, by monthly subculturing,  
 218 could be maintained in this state for up to 18 months.

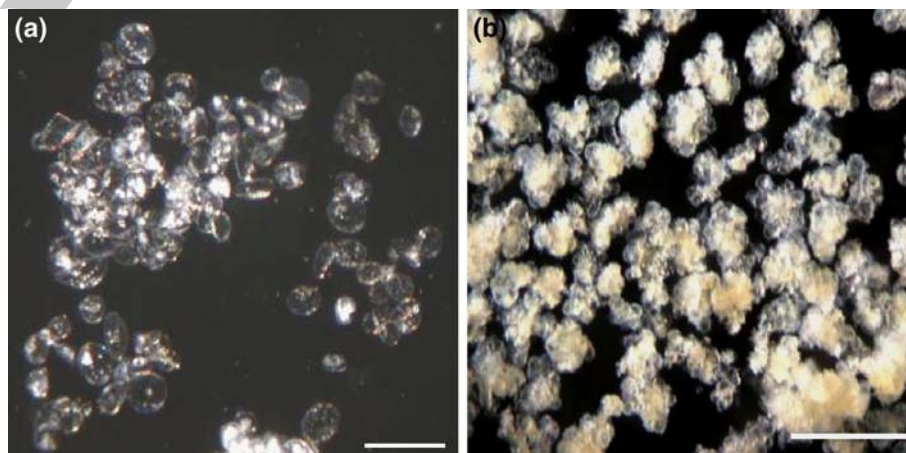
219 Glutamine concentrations higher than 800 mg/L did not  
 220 result in a further increase in fresh weight. The above  
 221 experiments indicated that the optimal medium for callus  
 222 proliferation on solid medium was half-strength MS con-  
 223 taining 1.0 mg/L TDZ, 0.5 mg/L 2,4-D and 800 mg/L  
 224 glutamine; this medium is henceforth referred to as solid  
 225 callus maintenance medium (CMM<sub>S</sub>).

## Initiation and characterization of suspension cultures 226

227 Suspension cultures were initiated by transferring approx-  
 228 imately 0.5 g embryogenic callus into liquid CMM con-  
 229 taining glutamine (100 mg/L) with continuous agitation at  
 230 90 rpm. When callus was taken directly from the original  
 231 explants (corm slices on callus induction media (CIM; Deo  
 232 et al. 2009) and placed in liquid CMM, some callus pieces  
 233 enlarged while others became necrotic after 2 weeks.  
 234 However, when callus was removed from the original  
 235 explants and placed on CMM<sub>S</sub> for 2 months with monthly  
 236 subcultures prior to transfer into liquid CMM, nearly all  
 237 inoculated calli formed suspensions. These callus pieces  
 238 increased in size by two-three fold 2 weeks after inocula-  
 239 tion into liquid medium and began to produce single cells  
 240 and small cell aggregates by the third week. Initially, most  
 241 of the cells released into the liquid were singular, large and  
 242 vacuolated (Fig. 1a), however, multicellular aggregates  
 243 containing cells with dense cytoplasm began to form with  
 244 subsequent weekly subcultures (Fig. 1b).

245 Suspension cultures contained two distinct cell types;  
 246 (1) spherical cytoplasmically dense cells with small vacu-  
 247 oles and numerous starch granules present as small multi-  
 248 cellular clumps and (2) elongated cells with large vacuoles,  
 249 which appeared transparent and contained very few or no  
 250 starch grains. Moreover, the cultures were heterogeneous  
 251 since they contained single cells, small multicellular  
 252 aggregates (0.1–0.5 mm diameter) and larger clumps (0.5–  
 253 1.0 mm diameter). Three to four months after initiation,  
 254 most suspension cultures produced cytoplasmically dense

**Fig. 1** Cell types present in suspension cell cultures of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. Initially, callus formed large vacuolated cells (a), but over time yellow, dense, small multicellular aggregates began to form (b). Scale bar 0.5 mm (a); 1 mm (b)





255 cell aggregates suitable for regeneration. However, after  
 256 7 months, the cell lines began to vary in their proliferation  
 257 rate and proportion of different cell types. For example,  
 258 some cell lines doubled in SCV within 2 weeks and con-  
 259 tained a high proportion of small, dense isodiametric cells  
 260 which were yellow in color. In contrast, other cell lines  
 261 took 1 month to double in SCV, contained a high propor-  
 262 tion of large, vacuolated cells and became pale yellow or  
 263 white. The latter type of cell line was regarded as having  
 264 poor regeneration capacity and was discarded.

265 Concentrations of glutamine greater than 100 mg/L (400  
 266 and 800 mg/L) were also trialed in suspension culture  
 267 media and, although this appeared to increase the prolif-  
 268 eration rate, cells cultured in this medium became necrotic  
 269 2 weeks after plating on RM. A similar negative effect on  
 270 regeneration was observed by reducing the concentration  
 271 of sucrose in liquid culture from 30 g/L to 20 g/L. Since  
 272 suspension cultures maintained in liquid CMM containing  
 273 100 mg/L glutamine and 30 g/L sucrose appeared to cope  
 274 best with the transfer from liquid to solid media, this  
 275 medium was used in all subsequent experiments and is  
 276 referred to as liquid callus maintenance medium (CMM<sub>L</sub>).

## 277 Regeneration and plant development

278 Despite their survival in the short term, suspension cells  
 279 plated on RM did not form embryos but, instead, prolif-  
 280 erated slightly then turned necrotic after 1–2 months  
 281 (Fig. 2a). Therefore, four embryogenesis media (EM) were  
 282 examined for their efficacy in maintaining cells in a healthy  
 283 state and inducing embryogenesis from suspension cells  
 284 (Table 3). Three weeks after plating cells on various EMs,  
 285 the large vacuolated cells became necrotic while the yellow  
 286 cell aggregates, consisting of small cytoplasmically dense  
 287 cells, proliferated and formed pro-embryogenic masses  
 288 (PEMs). Cell aggregates which were white formed soft,  
 289 white, watery callus. Globular structures began to form on

**Table 3** The effect of growth regulators on taro (*C. esculenta* var. *esculenta*, cv. CPUK) somatic embryo formation following transfer of suspension cells to embryogenesis medium (EM)

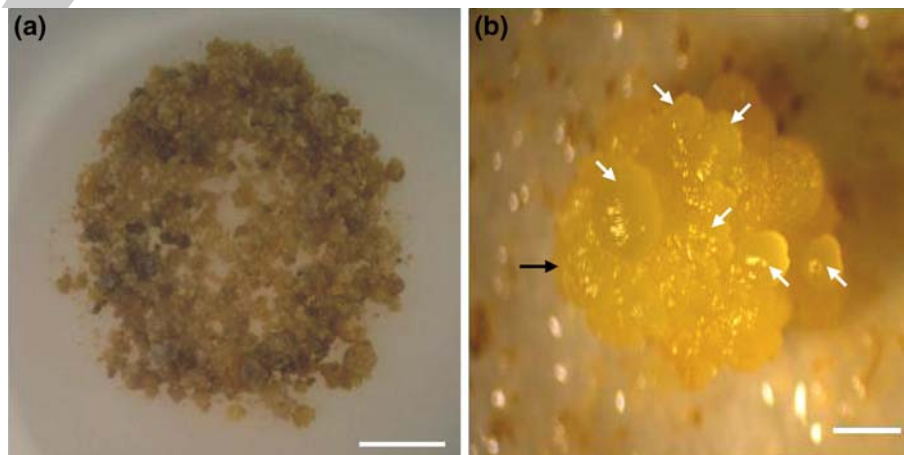
Embryogenesis medium	Plant growth regulators (mg/L)	Mean total number of somatic embryos produced per replicate
EM <sub>1</sub>	TDZ (1.0) + 2,4-D (0.5)	2.1 ± 0.82b
EM <sub>2</sub>	TDZ (0.1) + 2,4-D (0.05)	25 ± 4.3a
EM <sub>3</sub>	TDZ (0.01) + 2,4-D (0.005)	0.6 ± 0.43c
EM <sub>4</sub>	Zeatin (0.1) + NAA (0.05)	1.4 ± 0.62bc

Values with the means ± SE are derived from 10 replicate Petri dishes with 50 µL settled cell volume of suspension cells per replicate. Within a column, means followed by the same letters are not significantly different ( $P < 0.05$ )

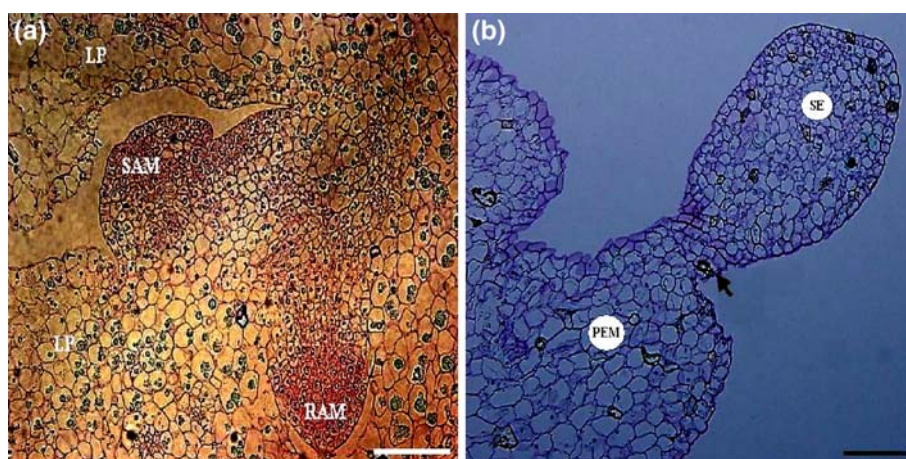
the surface of PEMs after 3 weeks and continued to do so for 2 months (Fig. 2b). Histological studies showed somatic embryos contained two meristems, presumably the shoot and root poles (Fig. 3a), and early in development were attached to PEM through a suspensor-like structure (Fig. 3b) indicating they were most likely derived from the surface cells of PEMs.

The highest rate of somatic embryo formation was from suspension cells plated on EM<sub>2</sub> (25 ± 4.3) (Table 3). On other EMs, the rate of somatic embryo formation was low with callus displaying a range of responses; cells proliferated profusely but with very few embryos formed (EM<sub>1</sub>), poor cell proliferation and necrosis (EM<sub>3</sub>) and cell proliferation as soft watery callus (EM<sub>4</sub>). To further increase the embryo formation rate on EM<sub>2</sub>, the effect of increased sucrose concentration and the addition of biotin were examined (Table 4). After 2 months on various EM<sub>2</sub> media, somatic embryos were transferred to germination medium (GM) and the percentage germination recorded. In general, the frequency of somatic embryo formation and the germination rate increased with increasing sucrose concentration.

**Fig. 2** Formation of PEMs and somatic embryos from embryogenic suspension cells of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. Suspension cells proliferated and turned necrotic on RM (a) whereas on EM they formed PEMs (black arrow) with globular, translucent embryo-like structures (white arrows) forming on the surface of PEMs (b). Scale bar 5 mm (a), 2 mm (b)



**Fig. 3** Histology of mature and immature somatic embryos derived from cell suspension cultures of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. **a** A mature somatic embryo with shoot apical meristem (SAM) between leaf primordia (LP), root apical meristem (RAM); **b** a globular somatic embryo (SE) attached to proembryogenic mass (PEM) via suspensor-like structure (black arrow). Scale bar 100  $\mu$ m



**Table 4** Effect of sucrose and biotin on the frequency of embryo formation and germination from embryogenic suspension cells of *C. esculenta* var. *esculenta* cv. CPUK following transfer to embryogenesis medium (EM)

Sucrose concentration in medium (g/L)	Mean total number of embryos produced per replicate	Total number of embryos transferred to germination medium	Total number of embryos germinated	% Germination
30	58 $\pm$ 3.6b	300	96	32
40	60 $\pm$ 4.8b	260	105	40
50	137 $\pm$ 15a	440	256	58
40 + Biotin (1.0 mg/L)	154 $\pm$ 19a	740	233	32

Values with the means  $\pm$  SEM are derived from 10 replicate Petri dishes with 50  $\mu$ L settled cell volume of embryogenic suspension cells per replicate. Within a column, means followed by the same letters are not significantly different ( $P < 0.05$ )

311 The addition of biotin to EM<sub>2</sub> containing 40 g/L sucrose  
 312 resulted in a significantly higher frequency of somatic  
 313 embryos which was comparable to the 50 g/L of sucrose but  
 314 with a lower germination frequency. Embryo formation was  
 315 non-synchronous with various stages of embryo develop-  
 316 ment being observed at the same time. When transferred to  
 317 GM, embryos were closely associated and difficult to sep-  
 318 arate without damage occurring. After 3 weeks on GM,  
 319 embryos began to enlarge and turn from translucent to  
 320 opaque. In the subsequent 2–3 months, they turned green  
 321 and germinated (Fig. 4a, 4b). At this stage individual plants  
 322 could be separated (Fig. 5a) and were transferred into  
 323 28 mL McCartney bottles containing 10 mL of half-strength  
 324 MS medium for further development (Fig. 5b). After  
 325 1 month in culture, all the plants reached a height of 6–8 cm  
 326 and appeared phenotypically normal.

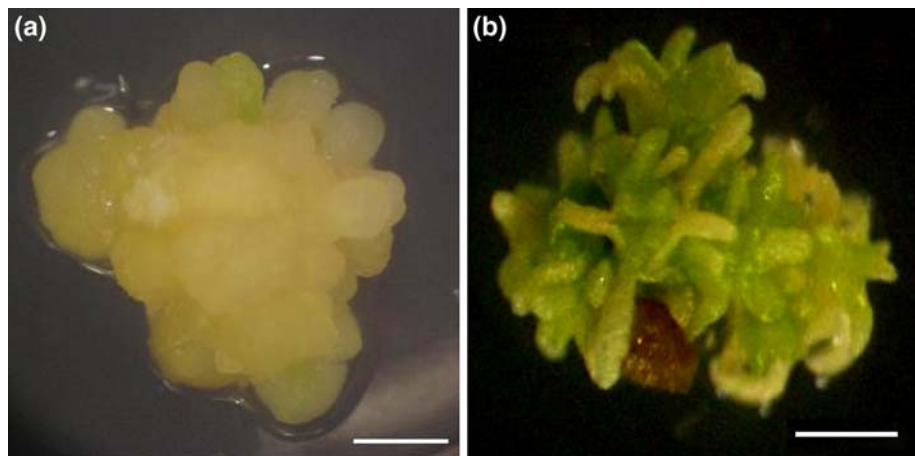
## 327 Discussion

328 Callus proliferation is an integral part of any efficient  
 329 regeneration system since it provides a continuous supply  
 330 of tissue thus reducing the requirement for initiating new

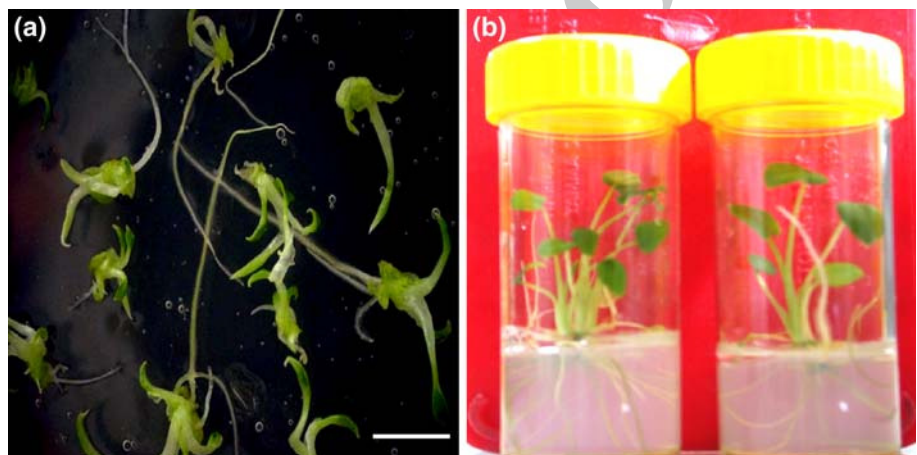
331 cultures. In this study, half-strength MS medium with  
 332 1.0 mg/L TDZ, 0.5 mg/L 2,4-D and 800 mg/L glutamine  
 333 was shown to be a suitable solid medium for callus  
 334 maintenance as it promoted proliferation whilst maintain-  
 335 ing embryogenic capacity. A combination of TDZ and 2,4-  
 336 D without glutamine induced callus proliferation, however,  
 337 approximately 40% of callus became soft, watery and non-  
 338 regenerable. The addition of glutamine at 800 mg/L to the  
 339 callus maintenance medium increased the proliferation rate  
 340 while at the same time maintaining the regeneration  
 341 capacity. Glutamine concentrations higher than 800 mg/L  
 342 appeared to have a negative effect and reduced both the  
 343 frequency of proliferation and the mean fresh weight of  
 344 callus. Glutamine readily increases the amount of available  
 345 nitrogen which enhances the synthesis of certain macro-  
 346 molecules or metabolites (Ogita et al. 2001) while main-  
 347 taining inorganic nitrogen at a low concentration. In this  
 348 study, the effect of glutamine was found to be dependent  
 349 on whether the media was liquid or solid. For example,  
 350 embryogenesis was inhibited in cells derived from liquid  
 351 medium containing 800 mg/L glutamine with all cells  
 352 turning necrotic after 2–3 weeks following transfer to  
 353 hormone-free medium. In contrast, the same concentration



**Fig. 4** Maturation and germination of somatic embryos from suspension cultures of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. Upon transfer to GM, embryo formation continued while the existing somatic embryos began to turn opaque and then green after 2 months (a). Germination commenced after 2 months (b). Scale bar 2 mm



**Fig. 5** Regeneration of taro plants from somatic embryos of taro (*Colocasia esculenta* var. *esculenta*) cv. CPUK. **a** Early germinating somatic embryos showing shoot and root formation, **b** Germinated somatic embryos after transfer to individual culture vessels. (Scale bar 2 mm)



354 in solidified callus maintenance medium did not appear to  
 355 be inhibitory even when transferred to hormone-free  
 356 medium. The inhibitory effect of glutamine on liquid-cultured  
 357 cells was avoided by reducing the concentration to  
 358 100 mg/L. While glutamine provides nitrogen in an  
 359 organic form, it is chemically unstable and degrades to  
 360 release ammonia (Barrett et al. 1997; Gorret et al. 2004). It  
 361 is possible, therefore, that at concentrations above 100 mg/  
 362 L too much glutamine or its degradation products were  
 363 made available to cells immersed in liquid media.

364 The inability to initiate suspension cultures using callus  
 365 taken directly from explants on callus initiation media may  
 366 have been due to (1) the shock from the physical isolation  
 367 of callus from the initial explants or alternatively, (2) the  
 368 characteristics of the callus at this particular stage of  
 369 development. Transferring callus to CMM<sub>S</sub> prior to CMM<sub>L</sub>  
 370 may have provided a transition step for the callus to pro-  
 371 liferate and allowed it to adapt to the different media  
 372 composition. The improved friability of callus cultured on  
 373 CMM<sub>S</sub> may also have contributed to the ability of cells to  
 374 dissociate when agitated in CMM<sub>L</sub>.

375 In general, highly prolific cultures tend to lose the ability  
 376 to regenerate more rapidly than slower growing cultures

(Ikeda-Iwai et al. 2002). In contrast, rapidly growing taro  
 377 suspension cells were found to be more regenerable than  
 378 slower growing cell lines. The highly prolific suspension  
 379 cultures doubled in cell volume fortnightly and contained a  
 380 large proportion of cells with embryogenic characteristics,  
 381 namely cells that were small, cytoplasmically dense, iso-  
 382 diametric in shape and often present in small multicellular  
 383 clumps. Such cultures were cream/yellow in appearance.  
 384 When plated on EM, the yellow cell aggregates formed  
 385 PEMs and SEs and the ability of cells derived from these  
 386 rapidly growing cell lines to regenerate persisted for over  
 387 12 months.  
 388

389 Somatic embryogenesis from callus has been reported in  
 390 *C. esculenta* var. *esculenta* using hormone-free media (Deo  
 391 et al. 2009). In this present study, regeneration from sus-  
 392 pension cells required successive steps. In contrast to callus  
 393 taken directly from corm slices (Deo et al. 2009), no  
 394 embryos formed on hormone-free medium using cells from  
 395 suspension culture. However, when suspension cells were  
 396 plated on embryogenesis medium (EM) containing 0.1 mg/  
 397 L TDZ and 0.05 mg/L 2,4-D, they proliferated and formed  
 398 PEMs with globular somatic embryos forming on their  
 399 surface. One of the critical events leading to the formation



of somatic embryos is the establishment of cell polarity, which can result from an auxin concentration gradient when callus is transferred to medium with low or no auxin (Souter and Lindsey 2000). Such a gradient may be established as a result of endogenous auxin synthesis or by the provision of exogenous auxin (Ribnicky et al. 1996). It would appear that taro suspension cells could not synthesize and accumulate the required level of endogenous IAA and/or other cellular metabolites required for embryo formation. Consequently, the application of very low concentrations of exogenous 2,4-D and TDZ was necessary. Both of these growth regulators have been reported to modulate endogenous auxin (Visser et al. 1992; Ribnicky et al. 1996; Panaia et al. 2004).

An increase in sucrose concentrations (up to 50 g/L) in the embryogenesis medium enhanced the frequency of somatic embryogenesis from suspension cells. A high frequency of embryogenesis at high sucrose concentrations has also been reported in maize (Kamo et al. 1985), cucumber (Lou and Kako 1995), sugar cane (Blanco et al. 1999; Gandonou et al. 2005) and melon (Nakagawa et al. 2001). At these concentrations, the action of sucrose is likely to be as an osmoticum or other developmental regulator rather than solely a carbon source. The combination of biotin (1.0 mg/L) with sucrose (40 g/L) increased embryo formation by 2.6-fold when compared with the numbers formed on 40 g/L sucrose alone. Biotin is important in carboxylation reactions and regulating genes involved in synthesis of some fatty acids, and development of plant embryos (Wurtele and Nikolau 1992). The stimulating effect of biotin on embryogenesis has also been reported in date palm (Al-Khayri 2001) and carrot (Wurtele and Nikolau 1992). Although the use of biotin in this present study increased the number of somatic embryos formed, the germination rate was lower than using 50 g/L sucrose alone indicating high sucrose was important for both embryo formation and maturation leading to a higher germination rate.

Following embryo development and early maturation, further maturation and germination was achieved by complete removal of 2,4-D and TDZ from the media while maintaining very low concentrations of BA (0.05 mg/L) and IAA (0.1 mg/L). However, the highest germination rate was 58% indicating that there is further scope for improving regeneration by modifications to EM and/or GM.

In summary, two effective callus maintenance media (CMM<sub>S</sub> and CMM<sub>L</sub>) for taro were developed and embryogenic callus could be proliferated for over a year without losing regenerability. The highly regenerable and rapidly growing nature of suspension cell cultures represents an ideal target tissue for the genetic transformation and mass propagation of this plant.

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