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Direct Somatic Embryogenesis and Organogenesis from Axillary Meristem in Taro (*Colocasia esculenta* var. *esculenta*)

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Abstract: This is a first report on direct somatic embryogenesis and organogenesis of taro (*Colocasia esculenta* var. *esculenta*) using axillary meristem explants. Best somatic embryogenesis was observed in cultures that were established on Murashige and Skoog (1962) (MS medium) containing 10 µM 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 2 µM thidiazuron (TDZ) and then transferred on medium with 5 µM TDZ. MS medium containing 3 µM gibberellic acid (GA) and 4.5% sucrose proved best for inducing germination in somatic embryos which converted into 20.0 ± 3.46 complete plantlets per embryo cluster per explant on ½ strength MS basal salts with 1% sucrose. Best organogenesis was observed in cultures that were established on MS medium containing 2 µM TDZ and then transferred on medium with 5 µM TDZ. Subsequent transfer of these cultures on MS medium with 5 µM indole-3-acetic acid (IAA) and 7.5 µM 6-benzylaminopurine (BAP) resulted in maximum shoot multiplication. MS medium containing 2 µM IAA proved best for inducing rooting in multiplied shoots. Both direct somatic embryogenesis and organogenesis resulted in mass and rapid production of taro plantlets which were acclimatized and field transferred. Vigorous plant growth and healthy corm production was observed in the field. This in vitro propagation method of taro through direct somatic embryogenesis and organogenesis is significantly reliable over prevailing methods available for other cultivars and provides sustainable means of quality taro production in the Pacific and the Caribbean region where non-availability of elite seedlings is a limiting issue.

Keywords: Axillary Meristem, Taro, In Vitro Propagation, Micronesian Region, Direct Somatic Embryogenesis, Direct Organogenesis

1. Introduction

Taro (*Colocasia esculenta*), a monocotyledonous, succulent, glabrous and perennial herb, is a staple food crop in many island nations in the Pacific and the Caribbean [15]. Grown for its edible corms, leaves and petioles; nutritionally, taro is rich in fiber, calcium, potassium, iron, vitamins A, B1, B2, and C [16]. Cooked taro leaves have the same nutritional value of spinach. The presence of main bulk in form of fine grained hypoallergenic starch makes taro corms easily digestible (98.8%). Low glycemic index of taro renders it as an excellent food for diabetics, who require glucose to be released into their bloodstream slowly [15].

Taro has attained a special economic and socio-cultural significance in the Pacific islands where in addition to being a significant constituent of every day diet, it is used in cultural dishes and traditional ceremonies [20]. *Colocasia esculenta* var. *esculenta* varieties that produce a large corm with few cormels are preferred over *Colocasia esculenta* var. *antiquorum* varieties with several small cormels [27]. Taro (*Colocasia esculenta* var. *esculenta*) is the first choice of most islanders because of its favorable corm texture, color, taste, and salt tolerance [25].

Flowering and viable seed production rarely occurs in taro [15, 19]. Therefore, traditionally it is propagated vegetatively by using setts, which consist of the lower 30 cm of the leaf stalk together with the top 2 cm of corms. However, such vegetatively propagated taro plants are genetically vulnerable

to pests and pathogens [15]. Moreover, relying mainly on setts obtained from parental stock does not ensure availability of sufficient planting material. Strict quarantine measures in the small island states, further limit the opportunities to procure planting materials required for sustainable production of desired taro cultivars in the region [25].

Meristem culture is increasingly being appreciated as a potential measure for mass propagation of planting material and germplasm conservation in dicotyledonous and monocotyledonous crops [5, 11]. Although taro regeneration by meristem cultures [5, 28, 29] and indirect embryogenesis through callus [7] has been reported previously, till date there is no report on direct somatic embryogenesis and organogenesis of *Colocasia esculenta* var. *esculenta* using axillary meristem explants. Development of an efficient in vitro propagation method for mass and rapid production of elite taro plantlets by this study has resulted in promoting sustainable cultivation of taro in the North Pacific region and laid a foundation for further research on taro.

2. Material and Methods

2.1. Plant Material

Healthy, young, 30-45 cm tall taro plants (*Colocasia esculenta* var. *esculenta*) collected from farmer's field were maintained in the greenhouse for one month, and then used to obtain explants comprising of corm top attached to the base of petiole.

These explants were thoroughly washed with running tap water and were surface sterilized by immersion in 70% (v/v) ethanol for 15 min followed by a treatment with 3% (v/v) sodium hypochlorite solution with 5 drops of Tween 20 for 30 min. Sterilized explants were then rinsed 5 times with sterile distilled water and were kept immersed in it until axillary meristem (axillary meristem including surrounding tissue of 1.0x1.0 cm size) from peripheral region of corm top were excised for in vitro culture establishment.

2.2. Culture Medium

Murashige and Skoog (1962) [17] medium (MS medium) was used in this study as a basal medium. All media contained 0.8% agar and 3% sucrose (or otherwise as specified). The pH was adjusted to 5.8 prior to autoclaving. Different concentrations and combinations of auxins, cytokinins and gibberellins were used as supplements. Data for non-responsive combinations or concentrations of various plant growth regulators, sucrose, and MS basal salts are eliminated.

2.3. Direct Somatic Embryogenesis

The direct somatic embryogenesis of taro was comprised of the following four phases: 1) Somatic embryo induction (Culture Phase 1, CP1); 2) somatic embryo development (Culture Phase 2, CP2); 3) somatic embryo germination (Culture Phase 3, CP3); and 4) complete plantlet development (Culture Phase 4, CP4) (Figure 1 and 2). In CP1, to induce somatic embryogenesis, axillary meristem explants were incubated on MS medium supplemented with 0-15 µM 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 0-3 µM thidiazuron (TDZ) for 4 weeks, and then three passages were given. Somatic embryo development was noticed in CP2, when highly responsive cultures of CP1 were transferred on MS medium containing 0-15 µM 2, 4-D and 0-7 µM TDZ for 4 weeks, after which two passages were given on the same media to induce somatic embryo maturation.

In CP3, to induce germination, mature embryos were transferred on MS medium containing 0-5 µM gibberellic acid (GA) and 3-6% sucrose for 4 weeks with two subsequent passages on same media. During CP4, the germinating embryos of CP3 were transferred on MS medium with half to full strength of MS basal salts and 1-5% sucrose, and three passages were given for further growth. The specific combinations of plant growth regulators used during these culture phases are shown in Table 1, 2 and 3. All cultures were initially kept at 22°C in the dark for somatic embryo induction in CP1, after which a 16-h photoperiod with 27 µmol m^{-2} s^{-1} light intensity was maintained during all other culture phases. The total incubation time for all four phases of somatic embryogenesis was 56 weeks, with the duration of CP1 being 16 weeks, CP2 and CP3 12 weeks each, and CP4 16 weeks, respectively. The frequency of plant conversion was examined by recording number of plantlets after in CP4. Each experiment was replicated three times with 30 explants per replication in CP1, and 15 cultures per replication in CP2.

2.4. Organogenesis

Three distinct phases were observed during organogenesis in taro: 1) Shoot initiation phase (SIP); 2) shoot multiplication phase 1 (SMP1); and 3) shoot multiplication phase 2 (SMP2) (Figure 3). During SIP, axillary meristem explants were inoculated on MS medium supplemented with 0-3 µM TDZ or 0-10 µM 6-benzylaminopurine (BA) for 4 weeks, and two passages were given. To induce organogenesis, the established cultures of SIP were given a transfer on MS medium augmented with 0-7 µM on which they were kept for 8 weeks. Multiple shoots induced during SMP1 were then transferred on MS medium augmented with 0-10 µM IAA and 0-10 µM BA for 4 weeks, and two passages were given for further growth and subsequent multiplication during SMP2. The total incubation time for all three phases of shoot organogenesis was 32 weeks with the duration of SIP and SMP2 being 12 weeks long, and SMP1 being 8 weeks long. Total number of plantlets produced was recorded after 12 weeks in SMP2. Table 4 shows various treatments of plant growth regulators given during all three phases. Every 4 weeks, the multiplication rate was recorded and each experiment was replicated three times with 30 explants per replication. A photoperiod of 12-h with 40µmol m^{-2} s^{-1} light intensity along with 28°C day and 24°C night temperature was maintained during all phases of organogenesis.

2.5. Rooting

MS medium augmented with 0-5 µM IAA was used to induce rooting in multiple shoots obtained through organogenesis (Figure 3). Fully elongated shoots of 4-5 cm height were transferred onto the rooting medium in groups of 20-25 shoots per culture (Figure 3c). After 4 weeks on rooting medium, percentage of rooting, number of roots per shoot and root length were recorded (Figure 3d) and each experiment was replicated three times with 100 cultures per replication.

2.6. Acclimatization

Complete taro plantlets with 8-15 cm root length, obtained via both direct somatic embryogenesis and organogenesis, were transferred into pots containing sterilized soil: vermiculate (1:1, v/v) mixture. These potted plantlets were kept in the greenhouse for first 4 weeks and then transferred into the screen house for next 4-8 weeks. Screen house was covered with 60% green color knitted shade cloth and temperature was maintained between 28-32°C with 50-55% humidity. During these phases of acclimatization, plants were irrigated once in 2 days with tap water and once per week with one-fourth, one-half, and full strength of MS basal salts. Survival of plants was recorded after 8 weeks. Each experiment was replicated three times with 100 plantlets per replication. After 4-8 weeks of ex vitro growth in the screen house, completely acclimatized plants were transferred in the nursery where they were kept until field transfer.

2.7. Statistical Analysis

Each experiment was replicated three times. A one-way analysis of variance was used to determine the level of significance between experimental treatments. Statistical significance of the results was determined using the least significant difference (LSD) test by Tukey (1953) [23] at 5% level of significance.

3. Results

3.1. Direct Somatic Embryogenesis

Best somatic embryogenesis was observed in axillary meristem explants that were incubated for a period of 16 weeks in CP1 on MS medium augmented with 10 µM 2,4-D and 2 µM TDZ (Table 1) and then were transferred on MS medium augmented with 5 µM TDZ for 12 weeks during CP2 (Table 2). Somatic embryos at various developmental stages such as nodular, globular, and scutellar stages were observed. During CP1, very small greenish-yellow nodular structures with smooth surface appeared on axillary meristem between bases of leaf petiole after 12 weeks of incubation which continued to enlarge for another 4 weeks. In CP2, greenish-yellow globular structures formed on the surface of the nodular structures from CP1. These globular structures eventually converted into clusters of somatic embryos (Figure 1c).

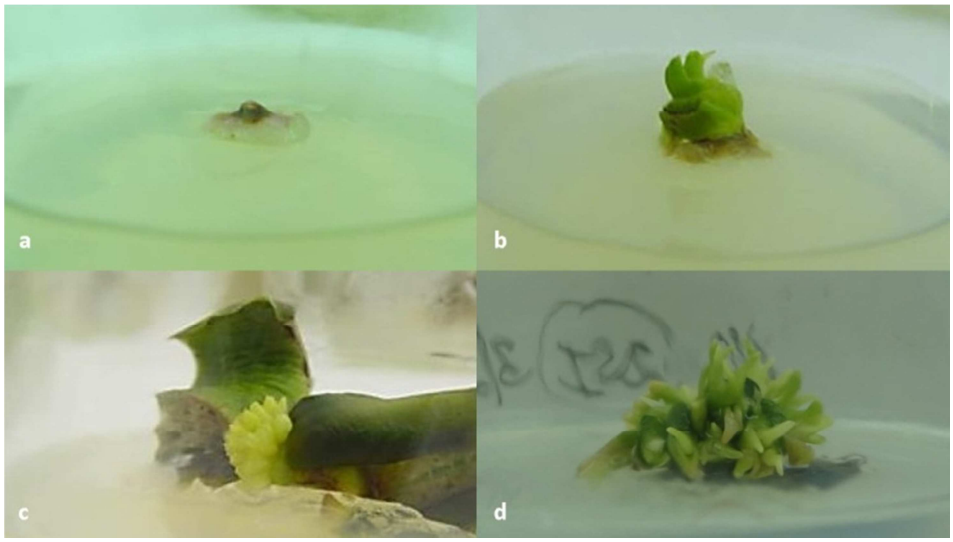

Figure 1. *Direct somatic embryogenesis (a) axillary meristem explant (CP1); (b) somatic embryo induction (CP1); (c) development of somatic embryos (CP2); and (d) germination of somatic embryos (CP3).*

The number of somatic embryos increased continuously up to 12 weeks with a maximum number of 25-30 embryos per embryo cluster initiated on each explant. Best embryo germination was observed when these embryo clusters were given a transfer on MS medium augmented with 3 µM GA and 4.5% sucrose for 12 weeks during CP3 (Figure 1d, and 2a; Table 3). During CP3, somatic embryos were clearly distinguished as they turned into white, opaque compact scutellar structures, and notch formation on scutellum was observed. Within 4 weeks on CP3, these embryos germinated as coleoptiles elongated and roots emerged. In CP4, subsequent speedy growth into complete plantlets was observed best on media with ½ strength MS basal salts and 1% sucrose (Figure 2b, c and d; Table 3). After 16 weeks on CP4, maximum 20.0 ± 3.46 complete plantlets were developed from the embryo clusters formed on each explant.

Figure 2. *Direct somatic embryogenesis (a) somatic embryo germination (CP3); (b), (c) and (d) complete plantlets (CP4).*

Table 1. *The effect of 2, 4-D and TDZ on direct somatic embryogenesis in Colocasia esculenta var. esculenta during CP1 after 16 weeks.*

Plant growth regulator(s) and concentration		Number of explants producing greenish -yellow nodular structures
2,4-D (µM)	TDZ (µM)	
5	0	0 ± 0.00
5	1	0 ± 0.00
5	2	2 ± 0.58d
5	3	0 ± 0.00
10	0	0 ± 0.00
10	1	5 ± 0.67c
10	2	18 ± 0.67a
10	3	11 ± 1.78b
15	0	0 ± 0.00
15	1	0 ± 0.00
15	2	1 ± 0.33e
15	3	0 ± 0.00

Values represent means ± SEM (Standard Error of the Mean), and are derived from 3 replications with 30 explants per replication. Values followed by the different letter in a column are significantly different at p≤0.05 according to Tukey (1953) LSD test range.

Table 2. *The effect of 2, 4-D and TDZ on direct somatic embryogenesis in Colocasia esculenta var. esculenta during CP2 in 12 weeks.*

Plant growth regulator(s) and concentration		Number of cultures producing greenish-yellow globular structures after 4 weeks	Number of cultures producing somatic embryos formed from globular structures after 8 weeks
2,4-D (µM)	TDZ (µM)		
0	0	0 ± 0.00	0 ± 0.00
0	1	4 ± 0.67c	1 ± 0.33c
0	3	8 ± 0.67b	2 ± 0.33b
0	5	14 ± 0.33a	14 ± 0.66a
0	7	4 ± 0.58c	1 ± 0.33c
5	0	0 ± 0.00	0 ± 0.00
5	1	2 ± 0.33d	0 ± 0.00
5	3	0 ± 0.00	0 ± 0.00
5	5	0 ± 0.00	0 ± 0.00
5	7	0 ± 0.00	0 ± 0.00

Values represent means ± SEM (Standard Error of the Mean), and are derived from 3 replications with 15 cultures per replication. Values followed by the different letter in a column are significantly different at p≤0.05 according to Tukey (1953) LSD test range. Data of non-responsive combinations or concentrations of 2, 4-D and TDZ are eliminated.

Table 3. *The effect of sucrose and GA on somatic embryo germination and complete plantlet development in Colocasia esculenta var. esculenta during CP3 and CP4.*

Sucrose concentration (%)	GA concentration (µM)	Number of cultures embryo germination observed in CP3 after 12 weeks	Number of cultures complete plantlet development observed in CP4 after 16 weeks
4.5	0	0 ± 0.00	0 ± 0.00
4.5	1	0 ± 0.00	0 ± 0.00
4.5	3	9 ± 0.33a	9 ± 0.33a
4.5	5	2 ± 0.33b	2 ± 0.33b
6.0	0	0 ± 0.00	0 ± 0.00
6.0	1	0 ± 0.00	0 ± 0.00
6.0	3	2 ± 0.33b	2 ± 0.33b
6.0	5	1 ± 0.33c	1 ± 0.33c

Values with means ± SEM are derived from 3 replications with 10 cultures producing somatic embryos per replication. Values followed by the different letter in a column are significantly different at p≤0.05 according to Tukey (1953) LSD test range. Data of non-responsive concentrations of sucrose are eliminated.

3.2. Organogenesis

In comparison to BA, TDZ was found to be more effective in inducing organogenesis in taro (Table 4). During first 4 weeks of SIP, small, green nodular structures appeared on the surface of axillary meristems inoculated on MS medium augmented with 1-3 µM TDZ. Out of these, media with 2 µM TDZ turned out to be the most promising treatment for inducing organogenesis. Upon transfer of these SIP cultures on MS medium augmented with 5 µM TDZ, the green nodular shoot buds proliferated into multiple shoots during SMP1 (Figure 3a). Excellent growth and elongation of SMP1 multiple shoots was observed when they were transferred on MS medium supplemented with 5 µM IAA and 7.5 µM BA during first 4 weeks of SMP2 (Figure 3b). After two subsequent passages, all multiple shoots converted into green shoots with 4-6 cm height within next 8 weeks of SMP2 (Figure 3c).

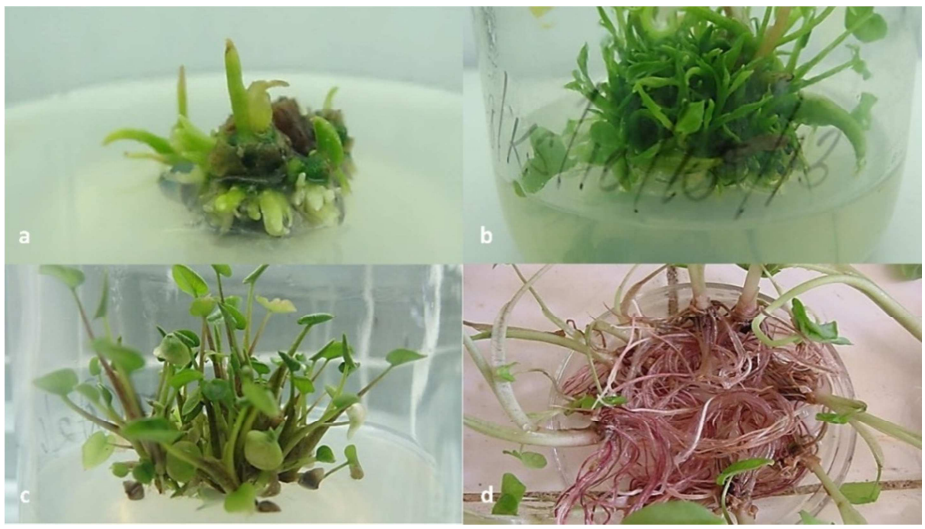

Figure 3. *Organogenesis and rooting (a) green nodular structures proliferated into multiple shoots; (b) elongation of shoots (c) multiple shoots on rooting medium; and (d) multiple shoots with fully developed roots.*

Table 4. *The effect of TDZ and BA on organogenesis in Colocasia esculenta var. esculenta during SIP, SMP1, and SMP2.*

SIP (12 weeks)			SMP1 (8 weeks)		SMP2 (12 weeks)		
Plant growth regulator(s) and concentration		Number of explants producing shoot buds	Plant growth regulator and concentration	Number of cultures showing multiple shoots	Plant growth regulator(s) and concentration		Number of cultures showing multiple shoots
TDZ (µM)	BA (µM)		TDZ (µM)		IAA (µM)	BA (µM)	
0	0	0 ± 0.00	-	-	-	-	-
1	0	11 ± 0.58c	5	6 ± 0.58c	5	7.5	5 ± 0.58c
2	0	20 ± 0.58a	5	22 ± 0.58a	5	7.5	26 ± 0.58a
3	0	14 ± 1.15b	5	9 ± 1.15b	5	7.5	7 ± 0.58b
0	1	0 ± 0.00	-	-	-	-	-
0	5	3 ± 1.20e	5	0 ± 0.00	-	-	-
0	10	8 ± 1.15d	5	3 ± 0.57d	5	7.5	1 ± 0.33d

Values with means ± SEM are derived from 3 replications with 30 explants per replication. Values followed by the different letter in a column are significantly different at p≤0.05 according to Tukey (1953) LSD test range. Data of non-responsive combinations or concentrations of TDZ or IAA and BA in SMP1 and SMP2, respectively are eliminated.

3.3. Rooting

Percentage of roots obtained in multiplied shoots was significantly different on various IAA concentrations (0, 1, 2 and 5 µM) (Figure 4). More than 90% rooting was recorded on MS medium augmented with 2 µM IAA within 4 weeks. After 4 weeks of incubation, 10.0 ± 2.89 roots with 8-15 cm length per shoot were observed (Figure 5a).

3.4. Acclimatization and Field Performance

More than 95% survival rate was observed in taro plants after 8 weeks of acclimatization in the greenhouse and screen house (total 12 weeks) when irrigated once in 2 days with tap water and once per week with one fourth strength of MS basal salts (Figure 6). These acclimatized plants exhibited healthy growth in the nursery (Figure 5b) where they were kept until they reached the height of 20-30 cm. Upon transfer of these fully acclimatized plants into the field, healthy and vigorous growth was observed (Figure 5c). After 10-12 months of field transfer, excellent yield of healthy taro corms (1.8-2.2 kg per main corm) was obtained (Figure 5d).

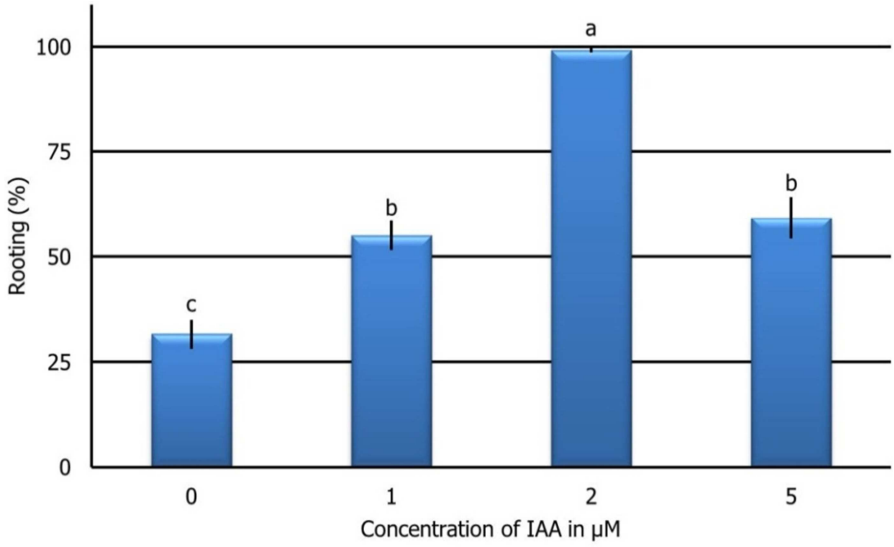

Figure 4. *The effect of IAA on in vitro rooting in Colocasia esculenta var. esculenta after 4 week's incubation on rooting medium. Values with means ± SEM are derived from 3 replications with 100 cultures per replication. Letters at the top of each bar are assigned on the basis of Tukey (1953) LSD test range (p≤0.05), where treatment that are significantly different, are assigned different letters.*

Figure 5. *Acclimatization, field transfer and harvesting (a) plantlets ready for hardening; (b) hardened plants in screen house; (c) plants in the field; and (d) harvested taro corms.*

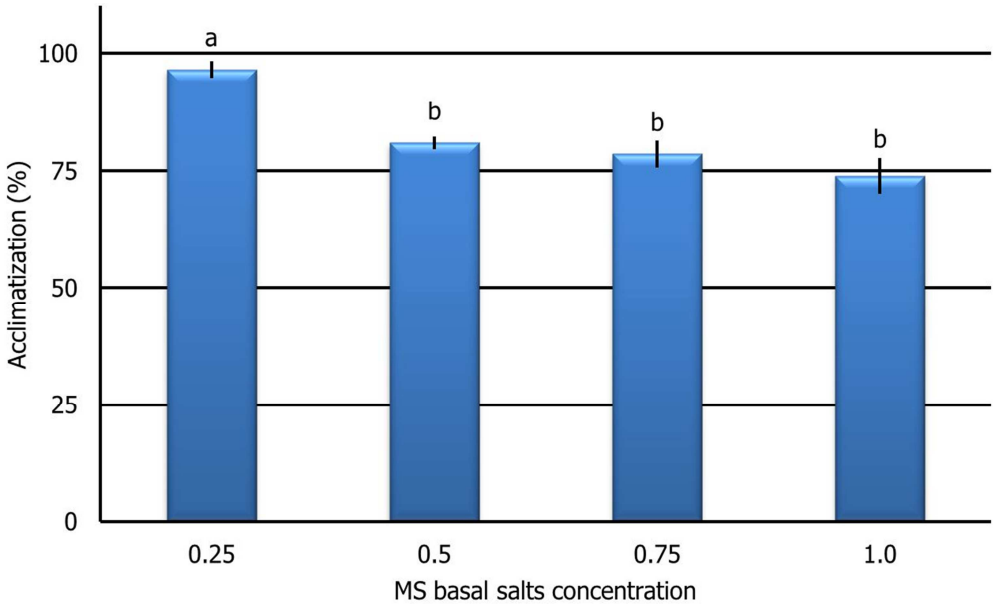

Figure 6. *The effect of MS basal salts concentration in irrigated water on plant survival rate in Colocasia esculenta var. esculenta within 8 weeks of acclimatization. Values with means ± SEM are derived from 3 replications with 100 plantlets per replication. Letters at the top of each bar are assigned on the basis of Tukey (1953) LSD test range ($p \leq 0.05$), where treatment that are significantly different, are assigned different letters.*

4. Discussion

This is the first report of direct somatic embryogenesis and organogenesis in taro using axillary meristem explants. Meristems have proved to be the most suitable explants for somatic embryogenesis in many plant species [11]. Abo El-Nil and Zettler [1] and Jackson et al. [8] used shoot-tips and meristems to initiate taro tissue culture. First callus formation and indirect plantlet development from axillary meristems of taro was reported by Yam et al. [28, 29]. Chand et al. [5] reported two-stage micropropagation technique for taro using sterile potting mix for plant maintenance at first stage and thereafter, inoculating meristems on a modified MS medium supplemented with TDZ. A method for indirect somatic embryogenesis in *Colocasia esculenta* var. *esculenta* cv. THA-07, CK-07 and CPUK from corm slices was reported by Deo et al. [7]. However, this method of indirect somatic embryogenesis was not reproducible in *Colocasia esculenta* var. *esculenta*. In our experiments, although yellow and fragile callus produced from taro corm slices, none of these calluses produced somatic embryos. Neither, organogenesis was observed.

In present study, somatic embryos originated directly from axillary meristems that were situated near the peripheral region of corm top. Somatic embryos at various developmental stages such as nodular, globular, and scutellar stages were observed. Combination of 2, 4-D with TDZ, played a decisive role in the induction of direct somatic embryogenesis in taro. Concentration of 10 µM 2, 4-D in combination with 2 µM TDZ in MS medium resulted in somatic embryo development in 60% explants during CP1. The initial somatic embryos produced at this medium were nodular in shape and greenish-yellow in color and were clearly distinct from the dark green nodular structures of organogenesis. Upon transfer on MS medium augmented with 5 µM TDZ during the CP2, these greenish-yellow nodular embryos converted into whitish-yellow embryos in 94% cultures. Similar to many other plant species, somatic embryo development in *Colocasia esculenta* var. *esculenta* occurred very soon after the elimination of auxin from the medium and by adding or increasing the concentration of cytokinins (in our study, TDZ) that promote somatic embryo maturation [12].

The high efficacy of TDZ for inducing somatic embryogenesis has already been demonstrated in several monocot and dicot plants [6, 7, 9, 13, 21, 22]. In many cases, TDZ induced embryogenesis was reported to be of higher efficiency and frequency as compared to other cytokinins or the combination treatments of auxins and cytokinins [3, 21, 26, 30]. TDZ either directly promotes somatic embryogenesis due to its own biological activity [26] or affects the endogenous ratios of endogenous auxins and cytokinins [21, 26]. TDZ has also been shown to induce accumulation of both endogenous auxins and cytokinins in legumes and monocots plants [4].

In this study, three levels of sucrose concentration (3%, 4.5%, and 6%) were studied for inducing germination in the somatic embryos during CP3. Best somatic embryo germination was observed on 4.5% sucrose along with 3 µM GA. This result aligns well with the previous studies which indicate that a relatively higher level of sucrose in the culture medium promotes maturation of somatic embryos [2, 12, 18, 24]. In this study, a sharp decline in percent germination of somatic embryos when sucrose concentration was increased to 6% with 3 µM GA indicates that the ratio of sucrose and GA concentration might also play a crucial role in inducing germination in the somatic embryos.

TDZ has also been reported to induce simultaneous formation of somatic embryos and shoots in *Phaseolus coccineus* [14]. However, in this study, use of TDZ alone

resulted in only organogenesis in taro. Lower concentration of TDZ (2 µM) found to be most effective for inducing organogenesis. The events leading to organogenesis appeared in three distinct phases: During initial SIP, small, dark green nodular structures appeared directly on the surface of apical meristem explants inoculated on MS medium with lower concentration of TDZ (2 µM). These nodular structures converted into light green multiple shoots upon transfer on MS medium with higher TDZ (5 µM TDZ) during SMP1. Replacement of TDZ with 5 µM IAA and 7.5 µM BA during SMP2 resulted in excellent elongation and conversion of light green SMP1 shoots into dark green shoots. The findings of this study are consistent with previous studies in which TDZ and several substituted pyridyl phenyl urea compounds have been demonstrated to stimulate meristem and shoot formation *in vitro* at low concentrations [10]. These compounds appear to have a strong cytokinin-like effect in a wide range of species, and even in the species that respond little to conventional, adenine-based cytokinins, although the compounds have molecular structures that are very different from those of adenine-based cytokinins [31].

5. Conclusion

This study reports for the first time, a useful and reliable in vitro propagation method of taro (*Colocasia esculenta* var. *esculenta*) via direct somatic embryogenesis and organogenesis. Development of this in vitro propagation method provides a means for mass, rapid production of elite taro plantlets of desired cultivars in the Micronesian region where procuring elite seedlings is a major hurdle in sustainable commercial taro cultivation. Mass production of in vitro taro cultures through this study also serves as a foundation for initiating further research in the areas of selection resistance, production of artificial seeds, and genetic transformation of taro in the region.

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