Callus Formation and Multiplication in Taro

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Summary

Callus was induced from shoot tip explants of taro (*Colocasia esculenta* Schott) using modified Nitsch medium containing major elements of Nitsch and minor elements and organic addenda of Ringe and Nitsch with 2% sucrose, 0.8% agar and 1 mg·liter⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenine (BA), each. The same growth regulators were used in the multiplication of callus. Furthermore, an auxin combination of 2,4-D and NAA at 1 mg·liter⁻¹ each have beneficial effects on the multiplication of callus.

By reducing the concentration of NH₄NO₃ from 720 mg·liter⁻¹ to 200 mg·liter⁻¹, the degree of callus formation and percent of explants forming callus increased. Low level of NH₄NO₃ was found to upgrade the formation and growth of callus.

Addition of 10% coconut water was beneficial to the induction of callus, on the other hand, it was not beneficial to the multiplication of callus but rather promoted root formation. Medium concentration had no significant effect on the formation and growth of callus.

Introduction

Callus of taro, a member of Araceae family, is difficult to induce from various plant parts (10). A report on callus formation from a Japanese cultivar of taro using different explants on a medium supplemented with 10⁻⁵ M BA and 10⁻⁶ M NAA showed some encouraging results with the percent of explants forming callus ranging from 25 to 78% (5). But at this rate of callus formation modifications of the basal medium should be considered since the rate is still very low to be adapted as a technique for taro callus propagation. Many researchers have tested various types of culture media in inducing taro callus but results are still unsatisfactory in considering an appropriate media, since callus formation appears to be variety dependent (4) and cultivar dependent (8). Linsmaier and Skoog (LS) medium (7) and Murashige and Skoog (MS) medium (12) are the widely used media in inducing taro callus, but usually explants on these media proceed to organogenesis resulting in the formation of shoots or plantlets.

Nitsch medium (13) was used by Geier (3) in the plant regeneration of *Anthurium scherzerianum* (Schott) of Araceae family, and he observed a sig-

nificant level of callus formation.

Plant regeneration from tissue and cell cultures is important in achieving rapid clonal multiplication, recovery of pathogen-free plants, preservation of valuable germplasm and induction of chromosomal and genic variation (6, 11, 20).

Since taro is propagated vegetatively, no breeding program has been established. That is why, present-day cultivars have existed for hundred of years without improvement. New and/or additional variations are indispensable for taro improvement. These variations can be induced in plantlets differentiated in cultured callus.

In this study, an investigation was undertaken in order to develop a model system of callus induction and multiplication of taro using Nitsch medium as the basal medium.

Materials and Methods

Apical shoots which emerged from taro (*Colocasia esculenta*) corms were excised and surface-sterilized after removing a few scale leaves. After the sterilization procedure, shoot tip explants were excised under a low-power dissecting microscope and explanted onto the culture medium containing major elements of Nitsch medium (13) and minor elements and organic addenda of Ringe and Nitsch medium (15) with 2% sucrose and 0.8% agar. This

medium was designated as modified Nitsch medium.

The pH was adjusted to 5.8 with HCl and NaOH before adding the agar. The medium was autoclaved at $1.2 \text{ kg}\cdot\text{cm}^{-2}$, 120°C for 15 min. Plant growth regulators were added before autoclaving. Explants were placed on 20 ml agar medium in test tubes covered with Morton caps. Incubation was normally at $25^\circ \pm 3^\circ\text{C}$ under dark condition since illumination was found to strongly inhibit the formation and growth of taro callus (8).

For the callus multiplication experiment, each piece of callus was divided to two or three pieces of not less than $3 \text{ mm} \times 3 \text{ mm}$. Necrotic parts were removed, and only the friable part on the base of the explant was used in the experiment. The pieces of callus were transferred to a fresh medium in a 200 ml Erlenmeyer flask. A square of aluminum foil (3 sheets) was used to seal the flasks.

The degree of callus formation and the degree of callus multiplication (DCM) were computed based on the average score using 4 level (0~3) scoring standard. The percent of explants forming roots and shoots was also measured.

1. Effects of 2,4-D and BA on callus formation

Shoot tip explants of cv. Egu-imo were placed on the basal medium with various concentrations of 2,4-D and BA, and the degree of callus formation and percent of callusing were observed after 40 days of culture.

2. Influence of NH_4NO_3 concentration on callus formation and multiplication

Shoot tip and callus explants of cv. Egu-imo were used in this experiment. Three types of culture media according to the adjusted NH_4NO_3 concentration were used in the experiment: 1) $0 \text{ mg}\cdot\text{liter}^{-1}$ ($\text{NH}_4^+ = 0 \text{ mM}$, $\text{NO}_3^- = 9.40 \text{ mM}$, ratio = 0), 2) $200 \text{ mg}\cdot\text{liter}^{-1}$ ($\text{NH}_4^+ = 2.50 \text{ mM}$, $\text{NO}_3^- = 11.90 \text{ mM}$, ratio = 1:4.8), 3) $720 \text{ mg}\cdot\text{liter}^{-1}$ ($\text{NH}_4^+ = 9.00 \text{ mM}$, $\text{NO}_3^- = 18.39 \text{ mM}$, ratio = 1:2.0).

2,4-D and BA were added at $1 \text{ mg}\cdot\text{liter}^{-1}$ each.

3. Relationship between the culture medium concentration and callus formation

Three cultivars namely, Egu-imo, Dotare and Takenoko-imo were used in this experiment. NH_4NO_3 concentration of the control medium (1X) was adjusted to $200 \text{ mg}\cdot\text{liter}^{-1}$. The medium con-

centration was adjusted into the following strength: 1) $1/2 \times$, 2) $1 \times$, 3) $2 \times$. 2,4-D and BA were added at $1 \text{ mg}\cdot\text{liter}^{-1}$ each.

4. Effect of coconut water on callus formation and multiplication

Three cultivars namely, Egu-imo, Dotare and Takenoko-imo were used in this experiment. NH_4NO_3 concentration of the culture medium was adjusted to $200 \text{ mg}\cdot\text{liter}^{-1}$. Coconut water from immature (green) nuts was used in the experiment. Coconut water was filtered and stored at -20°C . The coconut water was added to the medium at 10% (V/V) concentration prior to the pH adjustment.

5. Effect of different auxin combinations on the multiplication of callus

Callus of cv. Egu-imo was transferred on a fresh medium with various combinations of auxins, namely: 2,4-D + naphthalene acetic acid (NAA), 2,4-D + indolebutyric acid (IBA), 2,4-D + indoleacetic acid (IAA), NAA + IBA, NAA + IAA, IBA + IAA.

The auxins were added on the medium at $1 \text{ mg}\cdot\text{liter}^{-1}$ making a total of $2 \text{ mg}\cdot\text{liter}^{-1}$ of auxin in the medium. NH_4NO_3 concentration of the culture medium was adjusted to $200 \text{ mg}\cdot\text{liter}^{-1}$.

Results

After 3 weeks of culture, callus initiation was observed from the base of the shoot tip explants. After 60 days of culture, callus that formed from the explants was completely distinguishable. Callus that formed from the explants was of bubble type, a friable callus (Fig. 1A).

Observation through a scanning electron microscope (SEM) showed that the mass of cells was composed of small and large cells and had no epidermal tissue, signifying that the tissue induced in shoot tip culture is a callus and not a shoot primordium (Fig. 1B).

1. Effects of 2,4-D and BA on callus formation

Callus formation was observed in all media regardless of 2,4-D and BA concentrations (Table 1). The highest degree of callus formation and the percent of explants forming callus were recorded at 1.90 and 94% respectively in the medium with $1 \text{ mg}\cdot\text{liter}^{-1}$ 2,4-D and BA each. The addition of 2,4-D was beneficial to the formation of callus.

High level of 2,4-D and BA was detrimental to the formation of callus (Table 1).

2. Influence of NH_4NO_3 concentration on callus formation and multiplication

The degree of callus formation and the percent of explants forming callus were recorded highest in the medium with $200 \text{ mg}\cdot\text{liter}^{-1}$ NH_4NO_3 at 1.40 and 93.3%, respectively. The lowest level of callus formation was measured in the medium con-

taining $720 \text{ mg}\cdot\text{liter}^{-1}$ NH_4NO_3 (Table 2).

The degree of callus multiplication in the subculture was the same on the media with 0 and $200 \text{ mg}\cdot\text{liter}^{-1}$ NH_4NO_3 (Table 3). The degree of callus multiplication on the medium with $720 \text{ mg}\cdot\text{liter}^{-1}$ NH_4NO_3 was very low, at 0.5. The percent of explants forming hair root was highest in the medium with $720 \text{ mg}\cdot\text{liter}^{-1}$ NH_4NO_3 . The percent of explants forming root hairs increases with the concentration of NH_4NO_3 (Table 3).

In both experiments, the most number of explants showing browning, signs of necrosis, were observed on the medium not supplemented with NH_4NO_3 .

3. Relationship between the culture medium concentration and callus formation

Various morphogenetic responses were observed depending on the different strengths ($1/2 \times$, $1 \times$, $2 \times$) of modified Nitsch medium (Table 4). The degree of callus formation of all media was relatively high ranging from 1.5 to 2.2. Also, the per-

Fig. 1. (A) Taro callus induced from shoot tip explants on a modified Nitsch medium. (B) A taro callus observed from a scanning electron microscope (SEM).

Table 1. Effects of 2,4-D and BA concentrations on callus formation of taro.

Growth regulator ($\text{mg}\cdot\text{liter}^{-1}$)		No. of explants	No. of browning explants	Callus formation	
2,4-D	BA			%	Degree ^z
0.5	0	25	0	80	1.60
0	0.5	25	6	28	0.37
0.5	0.5	25	0	88	1.75
0.5	1	25	1	84	1.50
1	0.5	25	2	88	1.80
1	1	25	0	96	1.90
5	5	25	10	40	0.67

^z Degree of callus formation was based on the average score computed using 4-level (0; no callus formation, 1; slight callus formation, 2; moderate callus formation, 3; extensive callus formation) scoring standard.

Table 2. Influence of NH_4NO_3 concentration on taro callus formation.

NH_4NO_3 concn ($\text{mg}\cdot\text{liter}^{-1}$)	No. of explants	No. of browning explants	Callus formation		% of explants forming	
			%	Degree ^z	Roots	Shoots
0	15	5	60.0	1.3	40.0	6.7
200	15	1	93.3	1.4	26.7	0
720	15	3	80.0	0.9	20.0	0

^z Refer Table 1.

cent of explants forming callus was not affected by the concentration of the culture medium.

Differences on the responses among cultivars were observed, but there was no defined relationship between the medium strength and the percent of explants forming callus. Furthermore, the percent of explants forming shoots was not affected by the concentration of the culture medium. The percent of explants forming roots was high in 1 × strength.

4. Effect of coconut water on callus formation and multiplication

The degree of callus formation increased with the addition of coconut water (Table 5). The increase in the degree of callus formation with the addition of coconut water hastened the induction of callus from the explants, as the formation of callus on the medium supplemented with coconut water was observed earlier than that on the control medium.

There was no significant effect of coconut water

on the percent of explants forming callus.

There was no positive effect of coconut water on the multiplication of callus (Table 6). The addition of coconut water resulted in an enormous growth of root hairs covering the callus. Cultivar Dotare had the highest percentage of rooting at 90.9%.

5. Effect of different auxin combinations on the multiplication of callus

A very high degree of callus multiplication was obtained on the media with 2,4-D included on the combinations (2,4-D+NAA, 2,4-D+IBA, 2,4-D+IAA) (Table 7). The degree of callus multiplication recorded ranges from 2.1 to 2.3. The highest degree of callus multiplication at 2.3 was recorded on the medium containing 2,4-D and NAA.

In the media without 2,4-D, the degree of callus multiplication recorded ranges from 1.1 to 1.3. An enormous growth of roots was observed on these media especially on NAA+IAA with 96% of the explants forming roots.

Table 3. Influence of NH_4NO_3 concentration on the multiplication of taro callus.

NH_4NO_3 concn (mg·liter ⁻¹)	No. of explants	No. of browning explants	% of explants forming		DCM ²
			Roots	Shoots	
0	90	6	3.3	0	1.5
200	90	0	7.8	0	1.5
720	90	0	16.7	0	0.5

²DCM- Degree of callus multiplication. The degree of callus multiplication was based on the average score computed using 4-level (0; no growth, 1; slight growth, 2; moderate growth, 3; extensive growth) scoring standard.

Table 4. Relationship between the culture medium concentration and taro callus formation.

Cultivar	Medium concn	No. of explants	No. of browning explants	Callus formation		% of explants forming	
				%	Degree ²	Roots	Shoots
Egu-imo	1/2 ×	20	0	100	1.8	10	20
	1 ×	20	1	95	2.2	40	0
	2 ×	20	0	100	1.9	0	0
Dotare	1/2 ×	20	2	90	2.0	0	0
	1 ×	20	0	100	2.1	30	0
	2 ×	20	0	100	2.1	15	10
Takenoko-imo	1/2 ×	20	0	100	1.8	0	0
	1 ×	20	0	100	1.5	15	0
	2 ×	20	1	95	2.0	0	0

² Refer Table 1.

Shoot differentiation was only observed on the medium containing 2,4-D and NAA.

Discussion

Taro callus was induced in modified Nitsch medium with 2,4-D and BA. Results show that 2,4-D plays a very important role in the formation and multiplication of taro callus. Often, 2,4-D is used alone to initiate callus (2). The use of auxin combinations as a substitute for the usual auxin and cytokinin combination in the multiplication of taro callus was possible.

The many comparative studies of ammonium and nitrate as sources of nitrogen, undertaken prior to work with tissue and organ cultures, have revealed interesting differences between species and have emphasized the influence of environmental factors (19).

The formation and multiplication of taro callus were optimal on the medium with 200 mg·liter⁻¹ NH₄NO₃. In the study about the regeneration of *Anthurium scherzerianum* leaf segments, the NH₄NO₃ level had the most significant effect on callus and shoot formation (3). Also, Pierik and

Table 5. Modified Nitsch medium (1969) used for callus induction of different taro cultivars and the effect of coconut water on the formation of callus.

Cultivar	Without coconut water			With 10% coconut water		
	No. of explants	Callus formation		No. of explants	Callus formation	
		%	Degree ²		%	Degree
Egu-imo	16	87.5	1.6	17	88.2	2.1
Dotare	25	96.0	2.1	25	96.0	2.3
Takenoko-imo	25	92.0	1.5	25	92.0	1.9

² Refer Table 1.

Table 6. Effect of coconut water on the multiplication of taro callus.

Cultivar	Without coconut water				With 10% coconut water			
	No. of explants	% explants forming		DCM ²	No. of explants	% explants forming		DCM
		Shoots	Roots			Shoots	Roots	
Egu-imo	16	0	43.8	1.0	15	0	46.7	0.5
Dotare	16	0	62.5	0.3	11	0	90.9	0.5
Takenoko-imo	16	0	50.0	1.0	14	0	42.9	1.0

² Refer Table 3.

Table 7. Effect of different auxin combinations on the multiplication of taro callus.

Auxin combinations	No. of explants	No. of browning explants	% of explants forming		DCM ²	Remarks
			Roots	Shoots		
2,4-D+NAA	25	2	32.0	4.0	2.3	Dark yellow callus
2,4-D+IBA	25	1	28.0	0	2.1	Dark yellow callus
2,4-D+IAA	25	4	24.0	0	2.2	Dark yellow callus
NAA+IBA	25	3	64.0	0	1.1	Root elongation
NAA+IAA	25	0	96.0	0	1.3	Root elongation
IBA+IAA	25	0	88.0	0	1.2	Root elongation

Growth regulators (2,4-D, NAA, IAA, IBA) were supplemented at 1 mg·liter⁻¹ making a total of 2 mg·liter⁻¹ of auxins.

² Refer Table 3.

Steegmans (14) recommended callus induction on a medium with half strength of MS macroelements which means 825 mg-liter⁻¹ of NH₄NO₃.

The adjustment of NH₄NO₃ level to 200 mg-liter⁻¹ resulted in the low ratio of NH₄-N to NO₃-N from KNO₃ (950 mg-liter⁻¹). The beneficial effect of low ammonium/nitrate ratio on the formation of callus was observed in *Anthurium scherzerianum* (21).

This supports the idea that the beneficial effects of low NH₄NO₃ observed in *Anthurium*, also a member of family Araceae, have the same effects on the formation and multiplication of taro callus.

On the study of Geier (3) on *Anthurium scherzerianum*, high NH₄NO₃ level (720 mg-liter⁻¹) strongly accelerated root formation. But in our study, the increase of root formation due to high NH₄NO₃ was observed only on the callus multiplication experiment. A reverse effect was observed on the case of the callus induction experiment.

Generally, low medium concentration implies low level of available ions which may result to decreased growth of cells. On the other hand, high medium concentration implies a high level of ions or salts which is detrimental to cells due to salt sensitivity. Based on the results of this study, the culture medium concentration used in the experiment had no significant effects on the formation of taro callus.

Organic nutrient sources are effective for the proliferation of plant cells cultured in vitro (17, 18). In a liquid Knudson C (1946) medium added with coconut water and adenine, shoot tips of taro formed protocorm-like bodies, and when transferred to solidified MS medium with 10% coconut water, each explant produced 4 to 8 plantlets from the proliferated base of the shoot tip (10).

Taro callus was formed vigorously from taro shoot tip explants in the modified Nitsch medium supplemented with 2,4-D, BA and 10% coconut water. But, multiplication of callus was inhibited when subcultured in the presence of 10% coconut water. This may be due to the addition of coconut water, implying that whatever is good for callus induction may not be good for callus multiplication. Addition of coconut water seems to induce root differentiation.

A medium for taro callus induction was developed by Abo El-Nil and Zettler. This medium, a revised Schenk and Hildebrandt medium (16) con-

taining higher concentrations of PO₄⁻, NH₄⁺, Ca⁺⁺, Fe⁺⁺⁺, Zn⁺⁺ and B, is called AZ medium (1).

A study using this medium was conducted but the percent of explants forming callus was very low at 56.3% (9). Furthermore, when coconut water was added to the medium, no callus formation was observed from the shoot tip explants.

The above result implies that the modified Nitsch medium used in this study is the appropriate medium for taro callus induction and multiplication. Also, the technique introduced in this paper can be used in the improvement of taro cultivars through induction of genic or chromosomal variation in vitro.

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サトイモのカルスの形成および増殖

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摘 要

修正 Nitsch 培地を用いたサトイモの茎頂培養において、カルス形成が認められた。修正 Nitsch 培地の組成は Nitsch (1969) 培地の多量要素および Ringe・Nitsch (1968) の微量要素と有機化合物からなる。また、この培地にショ糖 2%, 寒天 0.8%, 2, 4-D $1 \text{ mg} \cdot \text{liter}^{-1}$ と BA $1 \text{ mg} \cdot \text{liter}^{-1}$ を添加した。同じ組成の培地はカルスの増殖にも有効であった。しかし、BA を加えない $1 \text{ mg} \cdot \text{liter}^{-1}$ 2, 4-D および $1 \text{ mg} \cdot \text{liter}^{-1}$ NAA の 2 種類のオーキシンの組み合わせでもカルスの増殖に

効果的だった。培地中の $720 \text{ mg} \cdot \text{liter}^{-1} \text{NH}_4\text{NO}_3$ 含量を $200 \text{ mg} \cdot \text{liter}^{-1}$ に減らすと、カルス形成程度およびカルス形成率は増加した。低濃度の NH_4NO_3 はカルスの増殖も促進した。

10% (V/V) の coconut water はカルス形成に有効であったが、カルス増殖には抑制的な影響を及ぼし、根の形成を促進した。修正 Nitsch 培地の濃度 (2, 1 および 1/2 倍) はカルス形成に有意な影響を及ぼさなかった。