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Induction of Somatic Embryogenesis in the Anther-Derived Calli of Bitter Gourd (*Momordica charantia* L.)

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ABSTRACT

Bitter gourd is an important crop being a valuable vegetable and a medicinal plant. There is a need of improving desired qualities through breeding programs. However, the limitation in conventional breeding method is high heterozygosity nature of the plant. Availability of homozygous bitter gourd breeding lines will overcome this problem. Anther culture is the most commonly use technique for producing homozygous lines. Present study was carried out to study the feasibility of inducing callus in the selected bitter gourd varieties, Mathale green and Thinnaveli white and breeding lines of *Palee* and TIA. Murashige and Skoog medium was used as the basal medium. Six media supplemented with different combinations of growth regulators were also tested for somatic embryogenesis in anther-derived calli. Results revealed that the variety Thinnaveli white was the best for inducing calli. Murashige and Skoog (MS) medium supplemented with 0.5 mg/L IBA, 0.2 mg/L GA_3 and 3 mg/L BAP was considered as the most responsive and cost effective for somatic embryogenesis induction in variety Mathale green and Thinnaveli white. The results revealed that the studies should be continued in order to produce the anther-derived homozygous lines to be used in the future breeding programs.

KEYWORDS: Anther culture, Bitter gourd, Callus induction, Primodia, Regeneration

INTRODUCTION

Bitter gourd *(Momordica charantia* L.) is an annual tendril herbage plant, belongs to family Cucurbitaceae. It can be grown in tropical and subtropical climates (Reyes *et al.*, 1994). It is widely distributed in China, Malaysia, India, Tropical Africa and America (Kirtikar and Basu, 1994). In Sri Lanka, it can be grown in all over the country during both seasons (Department of Agriculture, 2006).

Bitter gourd is an important and valuable vegetable containing high concentrations of ascorbic acid, vitamin A and C (Munsur *et al.*, 2007), iron and other minerals (Behera *et al.*, 2008). It has anti-microbial properties, hypoglycemic activity to reduce the blood glucose, anti-tumor, anti-spermatogenic and androgenic activities (Grover *et al.*, 2002; Khan, 1999; Xue *et al.*, 1998; Naseem *et al.*, 1998) that make the crop more valuable. Bitter gourd protein (MRK29) has been reported to be used as HIV inhibitor (Jiratchariyakul *et al.*, 2001).

Bitter gourd is a monoeceous bearing separate yellow color male and female flowers in the same vine at the leaf axils. Flowering occurs about 45 to 55 days after sowing. Due to cross pollination nature, they are highly heterozygous.

Improved hybrids can be developed through hybridization programs, however, homozygous lines are the prerequisite. Conventional breeding methods are time-consuming and requiring several generations to develop nearly homozygous lines and they are limited by space required for maintaining the field.

Anther culture is the most efficient and commonly used technique for rapid generation of doubled haploid plants (Massiah *et al.*, 2001). In this technique the developing anthers at a precise and critical stage are excised aseptically from unopened flower bud and cultured on a nutrient medium. The microspores within the cultured anther develop in to callus or embroyids that give rise to haploid plantlets either through organogenesis or embryogenesis. With a successful protocol, this technique shortens the breeding cycle (Andrea *et al.*, 2001) resulting complete homozygosity by doubling the chromosome number. There are number of factors that affect androgenesis, including genotype and physiological state of the donor plant, pollen developmental stage, pretreatment, culture medium, physical factors and chemical factors (Sopory and Munshi, 1996).

Production of haploids in bitter gourd through anther culture would allow breeders to use the superior line for improving the existing genotypes for different traits such as biotic and abiotic resistance, yield and fruit quality *etc.*

Although there are previous reports on callus induction of bitter gourd through anther

culture, no reports on callus induction, somatic embryogenesis and plant regeneration of local bitter gourd varieties. Thus, the present study was conducted to check the feasibility of callus induction and plant regeneration through somatic embryogenesis in selected bitter gourd genotypes.

MATERIALS AND METHODS

Experimental Location

This experiment was carried out at the Tissue Culture Laboratory of the Horticultural Crop Research and Development Institute Gannoruwa, Peradeniya during the period from January to May 2016.

Plant Materials

Four genotypes including two high yielding bitter gourd F_2 breeding lines TIA and *Palee*, and two recommended varieties by the Department of Agriculture, Mathale green (MG) and Thinnaveli white (TW) were used to induce androgenesis for future application in crop improvement programs. According to the regular observations of conventional experiments at Horticultural Crop Research and Development Institute, Gannoruwa, Peradeniya, TIA is a virus tolerant variety that produces good quality, long fruits. Mathale green and Thinnaveli white have shown acceptable agronomic characters.

Young flower buds in 6-8 mm length containing approximately 80% uninucleate microspores (Tang *et al.*, 2009) were collected from four genotypes grown in the experimental plots using standard agronomic practices. Flower buds were collected at 8-10 a.m.

Explant Preparation

After collecting the flower buds from the field, they were cleaned with a cotton wool wetted with 70% ethanol to remove impurities. After pretreating the flower buds at 4 °C for 24 hours in the dark, they were surface sterilized by dipping in 70% ethanol for 5 min followed by 2-3 rinses with sterile distilled water. Petals were removed aseptically and anthers were carefully excised with the forceps under the Laminar flow hood.

In Vitro Culture

The excised anthers from four varieties were inoculated on to the callus induction medium containing MS mineral salts and vitamins (Murashige and Skoog, 1962), supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 4 mg/L and 6-benzyl adenine (6-BA). Sucrose (30 g L^{-1}) was used as the carbon source. Anther-derived calli were transferred to six somatic embryogenic induction media containing different auxins and cytokinins as shown in the Table 1 supplemented with 30 g/L sucrose. The pH of all the media were adjusted to 5.8 and 0.8% (w/v) agar was added as a solidifying agent. The media were sterilized using autoclave for 20 min at 121 °C and 15 psi. Sterilized media were dispensed into the Petri plates (50x10 mm) each containing 15 mL of culture medium.

All the cultures were maintained at 25±1 °C whereas the cultured anthers were in the dark and the anther-derived calli were in the 16/8 photoperiod with 1800-2000 lux light intensity using white fluorescent light.

Table 1. Media composition tested for inducing somatic embryogenesis in the anther-derived calli

Medium	Growth regulators added to basal culture medium (mg/L)
MS-1	2 2,4-D + 0.5 IBA + 0.2 GA_3
MS-2	2.5 BAP + 0.2 IAA
MS-3	0.5 IBA + 0.2 GA_3 + 2.5 BAP
MS-4	0.5 IBA + 0.2 GA_3 + 1.0 BAP + 0.5 TDZ
MS-5	0.5 IBA + 0.2 GA_3 + 3 BAP
MS-6	0.5 IBA + 0.2 GA_3 + 1.0 BAP + 0.75 TDZ

BAP- Benzylaminopurine, 2,4-D-Dichlorophenoxyacetic acid, GA3- Gibberellic acid, IAA- Indole-3-acetic acid, IBA- Indole-3-butyric acid, TDZ- Thidiazuron; MS- Murashige and Skoog

Experimental Design

Completely Randomized Design (CRD) was used to check the response of four varieties for androgenesis induction and subsequent somatic embryogenesis. From each variety 20 anthers were cultured per petri plate on callus induction medium and 12 plates were used for each variety. Six regenaration media were tested using calli induced from anthers. Five calli from each variety were subcultured on to a food jar and six jars were used for each medium.

Data Recording

Observations were made on daily basis and the percentages of callus induction, somatic embryo formation, pigmentation and browning were calculated. Furthermore, in order to determine the time taken for regeneration cycle, number of days for callusing and embryo formation was recorded.

Statistical Analysis

The data were analyzed using General Linear Model (GLM) procedure of statistical analysis system using SAS 9.2 statistical software. Mean separation was done using Least Significant Difference (LSD) test at the 5% significance level.

RESULTS AND DISCUSSION

Effect of Genotype on Callus Induction

Callus induction was observed after one week of culture initiation. Analysis of variance on the callus induction rate indicated that the callus formation is significantly higher in the 2nd week than in 1st week (Table 2). A significant difference was not observed among the different genotypes within first week, however, Thinnaveli white gave rise to highest callus induction rate (69.32%; P<0.05) in the 2nd week.

Table 2. Mean callus induction percentage in four genotypes after different time periods of culture initiation

Variety	Callus induction rate (%)	
	1st Week	2nd Week
MG	39.45±3.34[a]	56.03±3.56[b]
Palee	33.98±4.75[a]	52.68±6.38[b]
TIA	38.10±4.10[a]	55.86±3.67[b]
TW	45.41±5.04[a]	69.32±4.23[c]

Means in a column with the same letters are not significantly different at P<0.05; n=48. MG- Mathale Green, TW- Thinnaveli White

Based on the results, genotype plays a critical role in androgenesis induction as reported by (Murovec and Bohanec, 2012).

Effect of the Culture Medium

Somatic embryo formation in anther-derived calli;

Mathale green and Thinnaweli white genotypes gave rise to highest somatic embryos of with 50.33% and 50.25% respectively; in MS-6 medium (P<0.05; Table 3). For other genotypes, *Palee* and TIA did not show any significant difference. Even though certain effect was observed among the culture media, further improvement is required for increasing the regeneration efficiency in the anther-derived calli.

Greening of callus

A significant difference in greening was not observed among the genotypes and media tested except in Thinnaveli white in MS-1 medium that gave the highest greening (63%; P<0.05; Table 4). Huge variation was observed in pigmentation development ranging in 8.5-37.0% in MG, 10.9-50.25% in *Palee*, 12.5-50.33% in TIA and 3.25-63% in TW. Furthermore, a negative relationship was observed between somatic embryo formation and greening. It has been reported that once the pigment development ceased the regeneration efficiency (Dalton, 1988).

Browning of callus

Browning causes to reduce the potential of inducing somatic embryogenesis in the culture anther-derived calli. A significant difference was observed among genotypes for callus browning in the tested regeneration media (Table 5). In *Palee* and Thinnaweli white a significant difference of browning was not observed in six tested culture media. In Mathale green highest browning (75%) was recorded in MS-1 medium that contained 2,4-D and lowest (8.5%) in MS-5 medium contained cytokinins and GA_3. In TIA the browning percentage was reached highest in MS-6 (50.25%) that contained TDZ with other cytokinins with GA3 and lowest (27.33%) in MS-3 medium.

Table 3. Effect of regeneration media on somatic embryo formation in anther-derived calli in bitter gourd

Media	Rate of somatic embryo formation (%)			
	MG	*Palee*	TIA	TW
MS-1	0±0.00[b]	25±0.25[a]	13±0.00[a]	0±0.00[b]
MS-2	0±0.00[b]	19±0.19[a]	12.5±0.12[a]	31.5±0.31[ab]
MS-3	42.37±0.10[a]	9.5±0.06[a]	29.33±0.09[a]	35.67±0.08[ab]
MS-4	37.75±0.06[a]	6.5±0.04[a]	27.17±0.08[a]	31.5±0.1[ab]
MS-5	42±0.07[a]	25.5±0.12[a]	28.25±0.08[a]	44±0.04[a]
MS-6	50.33±0.08[a]	6.5±0.06[a]	22.25±0.06[a]	50.25±0.11[a]

Means in a column with the same letters are not significantly different at P<0.05; n=144. MG- Mathale Green, TW- Thinnaveli White, MS- Murashige and Skoog

Table 4. Effect of regeneration media on greening from callus in bitter gourd

Media	Greening rate (%)			
	MG	*Palee*	TIA	TW
MS-1	31.5±0.31[a]	25±0.00[a]	12.5±0.12[a]	63±0.00[b]
MS-2	37.5±0.37[a]	31.5±0.06[a]	19±0.59[a]	19±0.06[ac]
MS-3	17.25±0.09[a]	34.75±0.18[a]	50.33±0.08[a]	21±0.07[ac]
MS-4	17.37±0.07[a]	50.25±0.18[a]	33.5±0.14[a]	33.67±0.10[bc]
MS-5	8.5±0.04[a]	19±0.06[a]	44±0.13[a]	9.5±0.06[a]
MS-6	4.33±0.03[a]	25.5±0.12[a]	38±0.01[a]	3.25±0.03[a]

Means in a column with the same letters are not significantly different at P<0.05; n=144. MG- Mathale Green, TW- Thinnaveli White, MS- Murashige and Skoog

Table 5. Effect of regeneration media on browning from callus in bitter gourd

Media	Browning rate (%)			
	MG	*Palee*	TIA	TW
MS-1	75±0.00^{b}	31.5±0.06^{a}	38±0.00^{ab}	56.5±0.56^{a}
MS-2	44±0.06^{ab}	37.5±0.12^{a}	38±0.00^{ab}	38±0.00^{a}
MS-3	28.5±0.07^{ad}	19.25±0.06^{a}	27.33±0.04^{b}	31.5±0.84^{a}
MS-4	23.5±0.08^{adc}	34.75±0.10^{a}	35.67±0.06^{ab}	42±0.07^{a}
MS-5	8.5±0.04^{c}	13±0.00^{a}	47±0.03^{a}	25.25±0.05^{a}
MS-6	14.83±0.04^{dc}	25.5±0.12^{a}	50.25±0.11^{a}	22±0.08^{a}

Means in a column with the same letters are not significantly different at P<0.05; n=144. MG- Mathale Green, TW- Thinnaveli White, MS- Murashige and Skoog

CONCLUSIONS

Thinnaveli white was performed well in callus induction medium supplemented with 1 mg/L 2,4-D and 2 mg/L BA. Selected microspore development stage and culture conditions were suitable for callus induction. Significant interaction effect was observed in media composition with variety. Thidiazuron showed a beneficial effect on somatic embryogenesis of TW.

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