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Research article

Mass Propagation System of Strawberry (*Fragaria* × *ananassa*) Microshoots by Liquid Shake Culture

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Abstract

The future of strawberry propagation and sales is very positive, so an efficient mass propagation system for strawberry (*Fragaria* × *ananassa*) was developed in this study. Strawberry leaf explants successfully induced green compact calli in semi-solid MS medium supplemented with 1 mg/l 2,4-D and 0.5 mg/l BA within 2 weeks. Subculture the calli in semi-solid MS medium supplemented with 1 mg/l TDZ and 0.2 mg/l 2,4-D produced 3.8 microshoots per callus within 49-56 days. These leaf-derived calli with adventitious shoots were cultured with 0.1 mg/l GA_3 and shaken at 120 rpm. The number of shoots increased from 15.3 shoots to 43.6 shoots per culture (100 ml) within 2 weeks, but all were hyperhydric shoots. Hyperhydric shoots recovered to normal shoots within 1 month when cultured on semi-solid MS medium with 8 g/l agar. Shoots formed roots on semi-solid MS medium supplemented with 0.5 mg/l IBA within 2 weeks. Rooted plantlets were acclimatized for 1 week before being transferred to the field at Bendito farm, where the strawberry plants were grown until maturity.

1. Introduction

Strawberry (*Fragaria* × *ananassa*) is one of the economically important fruits in Thailand, and it is mainly cultivated in the northern part of Thailand, a region of appropriate temperature and humidity for its propagation. Increased consumption of strawberry has not only been associated with its alluring color, scent and taste but also a source of useful compounds such as vitamin C, flavonoids, anthocyanins and other polyphenols [1]. The health benefits of strawberries have been the subject of many studies over recent decades. It has been demonstrated that this fruit exerts a wild range of biological activities such as antioxidant, cardioprotective effects and anti-inflammatory properties [2]. These activities have been attributed to the fruit's high level of polyphenols. Strawberry can be used as raw material for flavouring agent in food and beverage products.

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The cultivated strawberry is a dicotyledonous, perennial and low-growing herb. Conventional methods for vegetative propagation of strawberry are labor-intensive and offer low production of plants from a selected genotype. Tissue culture is one of the useful methods for mass propagation in a short time, preserving the genetic characteristics of the mother plants [3]. This method has been applied to many plants [4, 5], including the strawberry [6].

Earlier work on strawberry micropropagation was reported more than 30 years ago [7]. Strawberry leaf explants have been shown to have high regeneration rates [8-10]. In 1990, James *et al*. [11] reported the highest regeneration rates when using the youngest leaves explants. There have been many research studies on strawberry regeneration [12-15] but there are still problems with regeneration reported. For example, the highest percentage of shoot regeneration was only 4% [16], which was not suitable for commercialization.

The technique of microshoot culture in liquid medium, either under stationary [17] or shaking [18] conditions, allows the nutrient medium and plant growth regulators to stimulate and support the development and growth of multiple axillary buds. Continuous shaking medium causes the disappearance of apical dominance in microshoots, and thus leads to the induction and proliferation of axillary buds. Liquid cultures also dilute the toxic compounds (phenols) released by the shoots, producing culture oxidation and browning which usually occur in semi-solid medium. Nhut *et al*. [19] reported on the rapid mass shoot propagation of *Gladiolus* by liquid shake culture which produced 216 shoots in 50 ml MS liquid medium after three subcultures within 45 days. However, liquid culture can cause hyperhydricity of plantlets.

Therefore, the aims of this study were to determine the appropriate growth regulator for callogenesis and indirect organogenesis of strawberry leaf explants, establish the best suspension culture for mass propagation, facilitate recovery of hyperhydric shoots, promote root induction, acclimatize plantlets in peat moss, and finally transfer to the field.

2. Materials and Methods

2.1 Plant material and surface sterilization

Strawberry (*Fragaria* × *ananassa*) seeds were obtained from Bendito Farm, Mae Hong Son, Thailand. The seeds were surface sterilized with 20% clorox® containing 1 drop of Tween-20 per 100 ml for 20 min with occasionally shaking. The seeds were then removed and washed three times with sterile distilled water. Finally, the seeds were cultured on semi-solid MS medium [20] without plant growth regulators and incubated in a cultured room for 7 days in order to examine microbial contamination. The healthy seedlings were continually grown on MS medium for 2 months, after which the plantlets were used as a source of explants.

2.2 Callogenesis

In vitro leaf explants (0.5 cm long) were cultured on semi-solid MS media supplemented with 0.5, 1, 2 and 4 mg/l NAA or 2,4-D in combination with 0.5 and 1 mg/l BA. MS medium without plant growth regulators was used as control. The cultures were kept under 2000 lx light for 16 h per day at 25±2°C. The observed parameters for the callogenesis experiment were degree of callus formation, texture and color of callus, days for callus initiation and callogenesis percentage (%). Number of days of callus initiation was started from the inoculation of the explants and the other data was recorded after 4 weeks. Each treatment consisted of 15 replicates.

2.3 Shoot proliferation from leaf-derived callus

Light green compact calli from the callogenesis experiment were cultured on semi-solid MS medium supplemented with 1 mg/l 2,4-D and 0.5 mg/l BA for 4 weeks in order to use the explant in this experiment. Callus pieces (ca. 100 mg fresh weight) were subcultured on semi-solid MS media supplemented with 0.5, 1 or 1.5 mg/l TDZ in combination with 0.02 or 0.2 mg/l 2,4-D. MS medium without plant growth regulators was used as control. The cultures were kept under 2000 lx light for 16 h per day at 25±2°C. The observed parameters for shoot proliferation experiment were shoot regeneration percentage (%), days for shoot induction, and number of microshoots per explant. The number of days for shoot induction was started from the inoculation of the callus and the other data was recorded after 8 weeks. Each treatment consisted of 15 replicates.

2.4 Mass propagation of microshoots in liquid shake cultures

Leaf derived calli with adventitious shoots (ca. 3 g fresh weight per culture) were inoculated in 100 ml liquid MS media with different concentrations of GA_3(0.1, 0.5, 1 and 2 mg/l). MS liquid medium without plant growth regulators was used as control. The cultures were shaken at 120 rpm on an orbital shaker and kept under 2000 lx light for 16 h per day at 25±2°C. The observed parameters for mass propagation experiment were length of longest petiole, number of shoots per culture and the dry weight growth index. The data were recorded within 2 weeks. The dry weight growth index was measured using equation 1. Each treatment consisted of 15 replicates.

$$\text{Dry weight growth index} = \frac{DW_2 - DW_1}{DW_1} \quad (1)$$

Where: DW_1= Initial dry weight of calli with adventitious shoots (first day of culture) (g/culture)
DW_2= Final dry weight of microshoots (after 14 days of culture) (g/culture)

2.5 Effect of agar concentration on hyperhydric shoots

Two weeks old hyperhydric shoots (1 cm) from the mass propagation experiment were cultured on semi-solid MS media supplemented with different agar concentrations (8, 10, 12 and 14 g/l) without plant growth regulators. The cultures were kept under 2000 lx light for 16 h per day at 25±2°C. The observed parameters for this experiment were percentage of water content, fresh matter, dry matter and root formation percentage (%). The percentage of water content was measured using equation 2 [21]. The data were recorded within 4 weeks. Each treatment consisted of 15 replicates.

$$\text{Water content per shoot (\%)} = \frac{m_1 - m_2}{m_1} \; x \; 100 \quad (2)$$

where: m_1= Fresh weight (mg/shoot) and m_2 = Dry weight (mg/shoot)

2.6 Effect of growth regulators on the rooting of the plants

Healthy shoots (2 cm) without roots from the previous experiment were transferred to MS media supplemented with only NAA or IBA (0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/l). MS medium without plant growth regulators was used as control. The cultures were kept under 2000 lx light for 16 h per day at 25±2°C. The observed parameters for the root induction experiment were root formation percentage (%), days for root induction, average number of roots per culture and root morphology. The observation of number of day till the root induction was started following the

shoot inoculation and the other data was recorded 4 weeks later. Each treatment consisted of 15 replicates.

2.7 Acclimatization of plantlets

Regenerated plants, with strong stems and healthy root systems, were selected and thoroughly washed with tap water to remove any traces of agar before transferring into pots containing sterile peat moss, covered with a plastic bag that was gradually opened during the acclimation period. Plants were maintained in a culture room under 2000 lx light for 16 h per day at 25±2°C and watered every other day for 1 week. Acclimatized plants were transferred to the field of Bendito farm in Mae Hong Son.

2.8 Statistical analyses

Statistical parameters, such as percentage, mean, and standard deviation, were estimated using Microsoft Office Excel program and the data were analyzed by Duncan's multiple range test (DMRT) at $P \leq 0.05$ with SPSS ver. 23

3. Results and Discussion

3.1 Callogenesis

There was a wide range of variation in days for callus initiation, callogenesis percentage (%), degree of callus formation, callus characteristics (texture and color), depending on various concentrations of plant growth regulator application. In Table 1, the results showed that auxin (2,4-D and NAA) alone promoted callus induction and took a minimum of 7-14 days for callusing but the calli were creamy and soft in texture (Figure 1A). Friable callus occurred in MS medium with auxin alone because auxin plays a major role in controlling cell elongation, which consists of two interrelated processes; osmotic uptake of water and extension of the cell wall [22]. The combination of cytokinin (BA) and auxin (2,4-D and NAA) produced better results compared to the use of auxin alone. The combination of 1 mg/l 2,4-D and 0.5 mg/l BA produced maximum callogenesis percentage (100%) and minimum days for callus induction (7-14 days) and the calli were light green compact (Figure 1B). The auxin:cytokinin ratio plays an important role in the formation of cell phenotype, and in the regulation of organogenesis *in vitro* and plant morphogenesis [23]. Several researches reported that green calli with hard texture and good regeneration ability could be used for shoot proliferation [24-26]. In MS medium without any plant growth regulators, it was found that there was no callogenesis.

Our result was concomitant with Ara *et al*. [15] who found that the best response in callus induction of strawberry on MS medium was with 1.0 mg/l 2,4-D and 0.1 mg/l BA, 2 weeks after culture.

3.2 Shoot proliferation from leaf-derived callus

Diengngan and Murthy [27] found that TDZ was significantly better than BA in the performance of *in vitro* shoot response of strawberry nodal segments. Debnath [28] also reported that TDZ was more effective for plant regeneration from leaves of strawberry. In this experiment, shoot regeneration from leaf derived calli of strawberry cultured on MS medium with combination of 2,4-D and TDZ were summarized in Table 2. The results showed that green callus happened in every

treatment after 4 weeks, and adventitious buds appeared on these green calli resulting in microshoots within 49-56 days of culture. The best shoot regeneration percentage was observed on MS medium with 1 mg/l TDZ and 0.2 mg/l 2,4-D (73%), which gave the maximum number of shoots per callus (3.8 shoots/callus) as shown in Figure 2. Our result was concomitant with Passey *et al*. [29] who found the best response in shoot regeneration of strawberry on MS medium with 1.0 mg/l TDZ and 0.2 mg/l 2,4-D. However, all shoots obtained in the medium with TDZ and 2,4-D could not form any root.

Table 1. Effect of different concentrations of 2,4-D or NAA in combination with BA on callus induction of strawberry leaf explants after 4 weeks of culture

Plant growth regulators (mg/l)			Days for callus initiation	Callogenesis percentage (%)	Color and texture of callus	Degree of callus formation
2,4-D	**NAA**	**BA**				
0		0	-	0	-	-
0.5		0	7-14	80	CrFr	+
1.0		0	7-14	93	CrFr	++
2.0		0	7-14	93	CrFr	+++
4.0		0	14-21	80	CrFr	++
0.5		0.5	7-14	100	LGrCom	++
1.0		0.5	7-14	100	LGrCom	+++
2.0		0.5	7-14	100	LGrCom	+
4.0		0.5	7-14	100	LGrCom	++
0.5		1.0	14-21	93	LGrCom	++
1.0		1.0	14-21	93	LGrCom	+
2.0		1.0	14-21	93	LGrCom	+++
4.0		1.0	14-21	73	LGrCom	+
	0.5	0	7-14	100	CrFr	+
	1.0	0	7-14	53	CrFr	++
	2.0	0	14-21	73	CrFr	++
	4.0	0	14-21	80	CrFr	+
	0.5	0.5	14-21	73	CrCom	+
	1.0	0.5	14-21	100	LGrCom	+
	2.0	0.5	14-21	93	CrCom	++
	4.0	0.5	14-21	60	LGrCom	++
	0.5	1.0	21-28	80	LGrCom	++
	1.0	1.0	21-28	73	LGrCom	+
	2.0	1.0	21-28	100	LGrCom	++
	4.0	1.0	21-28	100	LGrCom	+

Note: Com = Compact, Fr = Friable, LGr = Light Green, Cr = Creamy
+ = Slight callus growth, ++ = Moderate callus growth, +++ = Massive callus grow, - = no callus

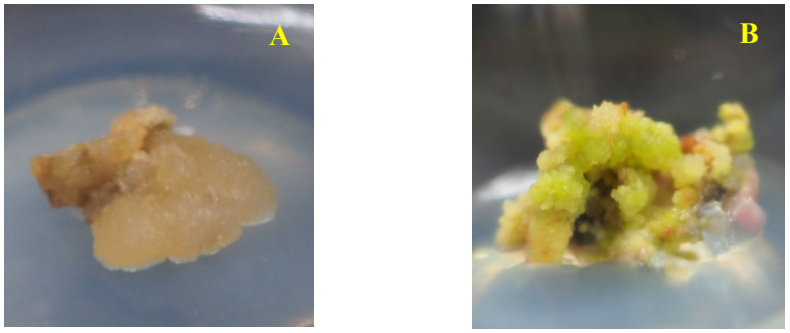

Figure 1. Texture and color of callus initiated from leaves explants in MS medium with different plant growth regulators. A) Creamy and soft texture callus in MS medium with auxin alone, B) Light green compact callus in MS medium with combination of auxin and cytokinin

Table 2. Effect of different concentrations of TDZ in combination with 2,4-D for shoot proliferation of strawberry (*Fragaria* × *ananassa*) after 8 weeks of culture

Plant growth regulators (mg/l)		Days for shoot proliferation	Shoot proliferation percentage (%)	Average number of shoots per callus (Mean±SD)
TDZ	2,4-D			
0	0	-	0	0e
0.5	0.02	49-56	13	0.6±1.59e
1.0	0.02	49-56	53	2.0±1.96bc
1.5	0.02	49-56	40	1.5±1.92cd
0.5	0.2	49-56	40	1.5±1.99cd
1.0	0.2	49-56	73	3.8±2.42a
1.5	0.2	49-56	33	1.4±2.32cd

Mean values with different letters in each column are significantly different according to Duncan's test at $P < 0.05$.

Figure 2. Shoot regeneration from leaf-derived callus cultured on MS medium supplemented with 1 mg/l TDZ and 0.2 mg/l 2,4-D after 49-56 days

3.3 Mass propagation of microshoots in liquid shake cultures

Suspension cultures provide an alternative and potentially more economic method for mass propagation. In this study, the influences of gibberellic acid (GA_3) on strawberry microshoot culture growth and development were investigated in suspension culture. Litwinczuk *et al*. [30] found that GA_3 significantly increased the number of axillary shoots of strawberry cultured in semi-solid medium. The present investigation is concomitant with the report by Litwinczuk *et al*. [30]. Table 3 showed that the number of shoots per culture in MS medium supplemented with GA_3 (0.1, 0.5, 1.0 and 2.0 mg/l) was significantly increased within 2 weeks of culture when compared to the MS medium without GA_3. Two mg/l GA_3 significantly increased the growth of microshoots (growth index = 25.62) which showed the highest elongation of petioles (1.23±0.08 cm). The highest number of proliferated shoots was found in MS medium supplemented with 0.1 mg/l GA_3 (43.6 shoots per culture) (Figure 3). However, the growth index was 14.06 because there was no elongation of petiole. In general, GA_3 at high concentration could increase elongation of shoot [31] but low concentration could increase the number of shoot formation [32]. For mass propagation of microshoots in liquid shake culture, the suitable GA_3 concentration was 0.1 mg/l. However, the proliferated shoots were hyperhydric with translucent, fragile leaves and without root formation. These characteristics are mainly caused by the high level of water available in cultures [33]. Hyperhydric shoots are the cause of considerable loss in commercial plant micropropagation. Reversion of shoot hyperhydricity was investigated in the next study.

Table 3. Effect of different concentrations of GA_3 for shoot proliferation in liquid shake cultures after 2 weeks of culture

Plant growth regulators (mg/l)	Longest petiole length (cm) (Mean±SD)	Average number of shoots per culture (Mean±SD)	Average Dry weight growth index (Mean±SD)
GA_3			
0	0.47±0.03d	15.3±1.52d	6.58±0.80c
0.1	0.59±0.08cd	43.6±1.15a	14.06±1.61b
0.5	0.73±0.07bc	32.6±1.52c	15.29±1.62b
1.0	0.81±0.09b	34.3±3.05c	23.67±2.44a
2.0	1.23±0.08a	38.0±3.00b	25.62±2.28a

Mean values with different letters in each column are significantly different according to Duncan's test at $P < 0.05$.

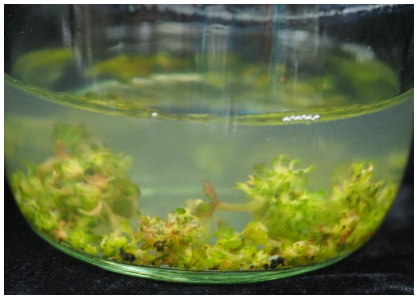

Figure 3. Mass propagation of microshoots in MS liquid medium supplemented with 0.1 mg/l GA_3 using a liquid shake culture system

3.4 Effect of agar concentration on hyperhydric shoots

The hyperhydric shoots derived from the previous experiment were cultured on MS media with different concentrations of agar. Casanova *et al.* [34] found that the hyperhydricity of adventitious shoots decreased when the agar concentration of MS medium was increased. The present investigation is concomitant with the report by Casanova *et al.* [34]. Plantlet hyperhydricity can be measured by water content and fresh matter of the plantlets. Table 4 showed that increasing the agar concentration in the medium resulted in decreasing the fresh matter and the water content of plantlets. We found that the water content of hyperhydric plantlets in the suspension culture was 92.5%. After 4 weeks of culture in 8g/l agar medium, 100% of the hyperhydric plantlets attained normal development. The normal plantlets had water content of 90%. However, we found that the higher the concentration of agar in the medium, the lower percentage of root formation in the plantlets (Table 4). Due to nutrient uptake limitation in higher agar concentrations, root production and growth of the plantlets was decreased. Thus, 8 g/l agar in the MS medium was the most suitable for reversion of shoot hyperhydricity.

Table 4. Effect of different concentrations of agar on the reversion of shoot hyperhydricity after 4 weeks of culture

Agar concentration (g/l)	Water content per shoot (%) (Mean±SD)	Average fresh weight matter (mg/shoot) (Mean±SD)	Average dry weight matter (mg/shoot) (Mean±SD)	Root formation percentage (%)
8	90±0.19a	347.0±7.19a	33.8±0.11a	53
10	88±1.63ab	214.5±5.48b	22.3±0.09a	46
12	87±1.24ab	211.9±9.76b	27.3±0.33a	20
14	82±1.13b	169.2±4.15c	30.0±0.43a	20

Mean values with different letters in each column are significantly different according to Duncan's test at $P < 0.05$.

3.5 Effect of growth regulators on the rooting of the plants

Based on frequency of rooting, number of roots and root germinated time, all treatments with IBA and NAA induced roots, but the highest mean number of roots (13.1±2.41) was recorded in the medium supplemented with 0.5 mg/l IBA, which started root initiation within 7-14 days of inoculation (Table 5). This indicated that NAA resulted in thicker and short roots in contrast to IBA containing medium. Callusing at the base of the shoots was observed in all MS medium that contained NAA. It was found that the higer the NAA concentration, the higher the callus formation. NAA mades roots shorter, which in turn caused less growth for roots in acclimatization medium.

3.6 Acclimatization of plantlets

Strawberry plantlets were grown *in vitro* with medium containing sugar and nutrients under high humidity and low light intensity in aseptic conditions, so they needed acclimatization in order to survive in the field. Usually, the stomata and root systems of cultured plants are poorly developed, so direct transfer to *ex vitro* conditions causes high mortality of the plantlets. In order to increase growth and reduce mortality in the plantlets at the acclimatization stage, the strawberry plantlets were grown in pots containing sterile peat moss, covered with plastic bags and maintained in the culture room for 1 week. After 1 week, the plastic bag covers were taken off, and 98% of the

acclimatized plantlets were able to be transferred to the field of Bendito farm, in Mae Hong Son. All the plants transferred to the field were grown until maturity as shown in Figure 4.

Table 5. Effect of different concentrations of NAA or IBA on root induction of strawberry plantlets (*Fragaria* × *ananassa*) after 4 weeks of culture

MS + Plant growth regulators (mg/l)		Days of root initiation	Root formation (%)	Number of roots/culture (Mean±SD)	Root morphology
NAA	IBA				
0		21-28	100	9.5±1.77bc	Thin, long
0.01		14-21	100	10.2±1.57ab	Thick, short, callus at base
0.02		21-28	90	8.9±1.09bc	Thick, short, callus at base
0.03		21-28	100	6.0±0.83def	Thick, short, callus at base
0.04		28-35	80	5.8±1.34ef	Thick, short, callus at base
0.05		21-28	70	5.4±1.41ef	Thick, short, callus at base
0.1		21-28	100	7.9±1.26cd	Thick, short, callus at base
0.2		21-28	90	6.1±1.75de	Thick, short, callus at base
0.3		21-28	70	6.3±1.00de	Thick, short, callus at base
0.4		21-28	60	1.3±0.21h	Thick, short, callus at base
0.5		21-28	80	2.1±0.64h	Thick, short, callus at base
	0.01	21-28	80	8.25±1.98cd	Thin, long
	0.02	7-14	60	7.8±1.40cd	Thin, long
	0.03	7-14	60	5.1±2.36efg	Thin, long
	0.04	14-21	60	3.1±0.77g	Thin, long
	0.05	14-21	50	5.3±1.37ef	Thin, long
	0.1	14-21	100	7.7±1.62cde	Thin, long
	0.2	7-14	100	9.3±1.34bc	Thin, long
	0.3	14-21	90	9.2±2.32bc	Thin, long
	0.4	14-21	100	10.0±1.38ab	Thin, long
	0.5	7-14	100	13.1±2.41a	Thin, long

Mean values with different letters in each column are significantly different according to Duncan's test at $P < 0.05$

Figure 4. Acclimatized strawberry plantlets transferred to the field of Bendito farm, in Mae Hong Son, after 2 months

4. Conclusions

From leaf explants to whole plant within 5 months through indirect organogenesis has proved to be a reliable method for mass propagation of strawberry, and especially in liquid shake culture. However, liquid media may produce hyperhydric plantlets which need a recovery process if they are to develop into normal plants. Somaclonal variation may occur since these plantlets were formed from callus. Further investigation is needed to better assess the performance and the genetic variations of these plantlets.

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