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Research article

Mulberroside A Accumulation in Mulberry Plantlets from *In Vitro* Culture and Hydroponic System

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Abstract

In this study, nodal explants cultured in MS medium supplemented with BAP 4mg/l and NAA 0.5 mg/l induced 3.33 shoots per explant within 4 weeks. The elongated multiple shoots were transferred to rooting media. The best root development was found on MS medium without PGRs within 10 days. The acclimatized plants were transferred to a deep-water hydroponic system with different concentrations of MS nutrient solution without sucrose addition. The results showed that mulberry plants cultivated in 1/8 MS nutrient solution gave the highest growth index. For mulberroside A determination, the results showed that mulberroside A accumulation in roots were higher than in shoots of mulberry plants both for *in vitro* culture and in the hydroponic system. In the case of *in vitro* culture, mulberroside A accumulation in roots was 20.61 mg/g DW, and in shoots was 11.98 mg/g DW. In hydroponic system, mulberroside A accumulation in roots was 17.58 mg/g DW, and in shoots was 2.34 mg/g DW. We also found that the higher concentration of MS nutrient solution, the more mulberroside A accumulation in roots of mulberry plants cultivated in the hydroponic system.

Keywords

plant tissue culture;
root induction;
secondary metabolites;
shoot multiplication;
soilless culture

1. Introduction

Mulberry (*Morus alba*), which is in the Moreaceae family and is a perennial plant grown all over the world, has its origins in India and China [1]. Mulberry leaves are used in silk production because they are rich in protein, carbohydrates and moisture [2]. Mulberry fruit is commonly consumed in mulberry-based products, such as juice, jam, wine, and natural food colorant [3]. Mulberry root has a number of bioactive compounds including resveratrol, oxyresveratrol, moracin, kuwanons G, and

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mulberroside A [4]. Resveratrol is an excellent antioxidant [5] and a prominent anti -inflammatory agent [6]. Oxyresveratrol is a natural compound found in mulberry, which has a wide range of biological activities such as its antioxidants [7], anti-diabetes [8] and anti-inflammatory roles [9]. Moracin, isolated from mulberry root, is an antioxidant, lipid peroxidation inhibitor, anti-Alzheimer's disease [10] and anti-glycation agent [11]. Kuwanons G is a flavonoid isolated from mulberry. It exhibits antimicrobial activity [12], antifungal activity [13], α-glucosidase activity [14] and protein tyrosine phosphatase 1B inhibitory activity [15]. Mulberroside A is a stilbene glycoside that shows many pharmacological including antioxidant activity [16], anti-tyrosinase activity [17] and anti-hyperlipidemic effects [18]. Mulberry roots from naturally grown mulberry trees take a long time to get to the harvesting stage. *In vitro* culture of mulberry can provide a large number of uniform plantlets in a short time, and these plantlets can be cultivated in hydroponic systems for mulberry growth and mulberroside A production. There is a lot of research on plant metabolite production by cultivating plants in hydroponic systems [19-22]. Hydroponically cultivated *Petroselinum crispum* showed significantly improved essential oil content when compared to pot cultivation [21]. Under hydroponic cultivation, *Datura innoxia* plants treated with MS nutrient solution improved plant growth and tropane alkaloid production [22]. Indoor hydroponic systems in a controlled environment allow the user to control important factors such as nutrients type, electrical conductivity, temperature, light intensity and the photoperiod, all of which play a vital role in plant growth, development and secondary metabolite accumulation [23, 24]. In this study, the micropropagation of mulberry through the *in vitro* culture of nodal explants was established, and the acclimatized plants were cultivated in a deep-water hydroponic system. The objective of the research was to study the accumulation of mulberroside A in mulberry plantlets produced in *in vitro* and hydroponic systems.

2. Materials and Methods

2.1 Plant material and surface sterilization

Mulberry tree (*Morus alba*) was grown in a garden of School of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. Nodal explants of this mulberry tree were collected and surface sterilized. Nodal segments of similar size (around 2.0 cm long) were initially cleaned with a dishwashing liquid followed by washing with running water for 40 min. The surfaces of nodal explants were sterilized with 70% (v/v) ethanol for 30 s and then sterilized with 1.2% (v/v) sodium hypochlorite containing 1 drop of tween-20 for 50 min followed by four washings with sterile distilled water for 5 min per wash.

2.2 Medium preparation and culture conditions for *in vitro* culture

The MS salts and vitamins that were described by Murashige and Skoog [25] containing 3% (w/v) sucrose and 0.8% (w/v) agar were used as the basal medium in all *in vitro* experiments. All plant growth regulators (PGRs) were added before the pH of the medium was adjusted to 5.6-5.8 using 0.1 N KOH or 0.1 N HCl. A total of 20 ml of MS medium were poured into glass culture vessel (diameter, 5.0 cm; height, 8.6 cm) before autoclaving at 121° C for 15 min. The cultures were incubated in culture room at 25±2°C under 2000 lux light for 16 h/day.

2.3 Shoot multiplication

The sterilized explants were cultured on MS medium supplemented with a combination of 6-benzylaminopurine (BAP) (1, 2, 3, 4 and 5 mg/L) and 1-naphthaleneacetic acid (NAA) (0, 0.5, 1, 1.5 and 2 mg/L). These media were used for multiple shoot induction experiments. Explants grown on MS medium without any PGRs were served as controls. After 4 weeks of culturing, the efficiency of PGRs on multiple shoot induction was evaluated by recording the number of shoots per explant, the average shoot length, and percentage of callus forming. The percentage of callus forming was measured with equation 1. Each treatment consisted of 6 replicates.

$$\text{Percentage of callus forming} = \frac{X_2}{X_1} \text{x } 100 \quad (1)$$

When: X_1 = Total number of cultured explants
X_2 = Total number of callus producing explants

2.4 Root induction

One-month-old regenerated shoots (2 cm long with leaves removed) obtained from shoot multiplication experiments were transferred to MS medium supplemented with various concentrations (1, 2 and 3 mg/l) of NAA or indole-3-butyric acid (IBA). The regenerated shoots grown on MS medium without any PGRs served as controls. Data were recorded after 3 weeks of cultivation. The efficiency of PGRs on root induction was evaluated by recording the date of root induction time, rooting percentage, the number of roots per explant, the average length of roots and percentage of callus forming. The percentage of callus forming was measured using equation 1. Each treatment consisted of 6 replicates.

2.5 Accumulation of mulberroside A in mulberry plantlets from *in vitro* culture

One-month-old regenerated shoots (2 cm long with leaves removed) obtained from the shoot multiplication experiments were transferred to MS medium without PGRs. After 4 weeks, *in vitro* plantlets with well-developed roots were assayed for mulberroside A content. The mulberry plantlets were cut into two parts (shoot and root). The shoots and roots were analyzed separately for mulberroside A content.

2.6 Plant acclimatization

In vitro plantlets with well-developed roots were removed from culture bottles and washed in running water to remove agar. They were then transplanted into glass culture vessels (700 mL) containing 500 mL of 1/24 liquid MS nutrient solution without sucrose. The vessels were covered with perforated plastic bags to maintain humidity for acclimatization. After 20 days of acclimatization, the plastic bags were removed and then left opened for 10 days. The acclimatized plants were used in the next experiment.

2.7 Effect of MS concentration on growth of mulberry plant in deep-water hydroponic system and mulberroside A accumulation

In this experiment, MS nutrient solution was used for plant growth in hydroponic culture. MS basal medium without sucrose in distilled water was adjusted pH to 5.6-5.8 by 0.1 N KOH or 0.1 N HCl and then diluted to 1/24, 1/20, 1/16, 1/12 and 1/8 with distilled water.

The acclimatized plants were transferred to deep-water hydroponic system in glass culture vessels containing different concentrations of MS nutrient solution (1/24, 1/20, 1/16, 1/12 and 1/8) without sucrose addition and cultivated for 4 weeks. They were cultivated in a culture room at 25±2°C under 2000 lux light for 16 h/day. Data were recorded after 4 weeks of cultivation. The efficiency of MS nutrient solution concentration on growth of mulberry plants and mulberroside A accumulation were evaluated by recording the fresh weight growth index, the average shoot length and mulberroside A content in shoots and roots. The fresh weight growth index was measured using equation 2. Each treatment consisted of 6 replicates.

$$\text{Fresh weight growth index} = \frac{\text{FW2} - \text{FW1}}{\text{FW1}} \quad (2)$$

When: FW1 = Initial fresh weight of mulberry plant (first day of culture) (g)
FW2 = Final fresh weight of mulberry plant (after cultivation) (g)

2.8 Mulberroside A determination

Shoot and root samples from *in vitro* culture and hydroponic system were dried in a hot air oven at 70°C for 12 h and then ground into powder. Powder (0.5 g) was soxhlet extracted with 200 ml of methanol for 2 h and then evaporated by rotary vacuum evaporator. After extraction, the crude extract was dissolved in 50 mL absolute methanol, and then filtered through 0.45 µm filter before HPLC analysis.

The crude extract were analyzed by high-performance liquid chromatography (HPLC, MiniLC-80, USA). The analysis was performed with an ROC C_{18} column (4.6 mm x 150 mm, 5µm; Restek, USA). The mobile phase consisted of 60% methanol and 0.1% acetic acid. The flow rate was set at 1 ml/min and injection volume was set at 20 µL. The UV-VIS detector was set at 320 nm [23]. Each sample was examined in triplicate. The concentration of mulberroside A was calculated from the calibration curve for standard mulberroside A.

2.9 Statistical analysis

The data were generated using IBM SPSS Statistics version: 28.0.0.0 (190). One-way analysis of variance (ANOVA) was performed to analyze differences of the efficiency of PGRs on shoot multiplication, the efficiency of PGRs on root induction and the mulberroside A content, and compared with Duncan's new multiple range test (DUNCAN) (*P=0.05*).

3. Results and Discussion

3.1 Effects of different concentrations of BAP and NAA on shoot multiplication

Nodal explants inoculated on MS medium with PGRs increased multiple shoot production as compared to MS medium without PGRs (Table 1). After 4 weeks of cultivation, the average shoot

length was not significantly different in all treatments (1.60 to 2.75 cm) (Table 1). The highest shoot number was 3.33 shoots per explant in treatment S17, S22, and S23 (Table 1). Callus was induced in all treatments but MS medium without PGRs had the lowest percentage of callus forming (50%) (Table 1). Most of the calli were yellow, small, moist and soft, and occurred at the base of the nodal explants. We found that nodal explants inoculated on MS medium supplemented with 4 mg/L BAP and 0.5 mg/L NAA (treatment S17, Figure 1) showed the most significant difference in the number of shoots per explant (Table 1). This result was similar to several reports [26, 27]. Sajeevan *et al*. [27] reported that MS medium supplemented with 1 mg/L BAP, 0.1 mg/L TDZ, and 0.25 mg/L NAA showed a maximum regeneration (85.67%) and maximum number of shoots per explant (7.33 shoots per explant). However, the number of shoots in our results were less than those found by Sajeevan *et al*. [27] probably due to unsuitable PGRs and their concentrations. So, we would use other PGRs and different combinations of them for a better result.

Table 1. Effect of BAP and NAA on shoot multiplication of mulberry nodal explants. Data were taken 4 weeks after culturing.

No. of treatments	Plant growth regulators (mg/L)		Average shoot length (cm)	Average no. of shoots per explant	Callus forming (%)
	BAP	NAA			
Control	0	0	1.60±0.36^{a}	1.00±0.00^{b}	50
S1		0	2.03±0.42^{a}	2.00±1.00^{ab}	100
S2		0.5	2.07±0.60^{a}	2.67±1.53^{ab}	100
S3	1	1	2.67±0.29^{a}	2.67±0.58^{ab}	100
S4		1.5	1.83±0.29^{a}	2.67±1.53^{ab}	100
S5		2	2.60±0.26^{a}	1.67±0.58^{ab}	100
S6		0	2.63±1.25^{a}	2.33±0.58^{ab}	100
S7		0.5	1.90±1.15^{a}	3.00±2.00^{ab}	100
S8	2	1	1.53±1.08^{a}	2.00±1.00^{ab}	100
S9		1.5	2.23±0.68^{a}	2.00±0.00^{ab}	100
S10		2	2.23±0.46^{a}	1.33±0.58^{ab}	100
S11		0	2.57±0.51^{a}	2.67±2.89^{ab}	100
S12		0.5	1.73±0.75^{a}	2.00±1.00^{ab}	100
S13	3	1	2.33±0.35^{a}	2.67±0.58^{ab}	100
S14		1.5	1.67±0.29^{a}	2.67±1.53^{ab}	100
S15		2	1.67±0.76^{a}	1.00±0.00^{b}	100
S16		0	2.75±1.77^{a}	3.00±2.83^{ab}	100
S17		0.5	2.50±0.17^{a}	3.33±0.58^{a}	100
S18	4	1	2.60±0.36^{a}	3.00±0.00^{ab}	100
S19		1.5	2.27±0.25^{a}	1.67±1.15^{ab}	100
S20		2	2.03±0.35^{a}	2.33±0.58^{ab}	100
S21		0	2.50±0.00^{a}	2.67±0.58^{ab}	100
S22		0.5	2.50±1.32^{a}	3.33±1.53^{a}	100
S23	5	1	1.83±0.29^{a}	3.33±0.58^{a}	100
S24		1.5	1.67±0.29^{a}	1.00±0.00^{b}	100
S25		2	1.73±0.25^{a}	2.33±0.58^{ab}	100

Data represents the mean±standard error. The same letter indicates no statistical differences at $p = 0.05$ according to the Duncan's new multiple range test.

Figure 1. Four weeks old multiple shoots from nodal explants of mulberry on MS medium supplemented with 4 mg/L BAP and 0.5 mg/L NAA

Aroonpong and Chang [28] suggested that BAP alone showed a positive effect on the multiple shoot production of mulberry plantlets. Moreover, the combination of PGRs was more efficient in inducing multiple shoots than individual ones [29, 30]. For this reason, MS medium supplemented with combination of 4 mg/L BAP and 0.5 mg/L NAA (treatment S17) was used for shoot multiplication in this research.

3.2 Effects of different concentrations of NAA or IBA on root induction

Shoots without roots from the last experiment were subcultured in rooting medium with different concentrations (1, 2 and 3 mg/L) of NAA or IBA. After 3 weeks of culture, the percentage of rooting ranged from 0 to 50% (Table 2). No rooting was induced in media treatment R2, R3, R5 and R6. The highest percentage of rooting was on MS medium without PGRs (50%) and root induction time was 10 days after inoculation (Table 2). The highest number of roots was 6.0 roots per explant on MS medium supplemented with 1 mg/L IBA (Table 2). The highest length of roots was 2.83 cm on MS medium without PGRs (Table 2). Callus was induced in all treatments but MS medium without PGRs had the lowest percentage of callus forming (50%) (Table 2). Most of the calli were yellow, small, moist and soft, and occurred at the base of the shoot explants.

Table 2. Effects of different concentrations of NAA or IBA on root induction. Data were taken 3 weeks after culturing.

No. of treatments	Plant growth regulators (mg/L)		rooting (%)	Root induction time (day)	Average no. of root per explant	Average length of roots (cm)	Callus forming (%)
Control	-		50	10	4.67±2.89^{b}	2.83±0.76^{a}	50
R1		1	33	15	1.00±0.00^{c}	0.25±0.07^{c}	100
R2	NAA	2	0	-	-	-	100
R3		3	0	-	-	-	100
R4		1	33	14	6.00±1.41^{a}	1.75±0.64^{b}	100
R5	IBA	2	0	-	-	-	100
R6		3	0	-	-	-	100

Data represents the mean±standard error. The same letter indicates no statistical differences at $p = 0.05$ according to the Duncan's new multiple range test.

In this study, our results were similar to the results of Aroonpong and Chang [28], who was shown that auxins were not necessary for inducing mulberry roots. Moreover, auxins also induced more callus forming at the base of the shoots and inhibited the root formation by interfering with the connection between shoot and root growth [31, 32]. Therefore, MS medium without PGRs was used for root induction.

3.3 Accumulation of mulberroside A in mulberry plantlets from *in vitro* culture

Shoots without roots from the shoot multiplication experiment were subcultured in MS medium. After 4 weeks of culture, the shoots and the roots were assayed for mulberroside A content. The result showed that the roots contained more mulberroside A than the shoot parts, as shown in Table 3. Mulberroside A content in shoots was 11.98 mg/g DW and mulberroside A content in roots was 20.61 mg/g DW. Our results were similar to the results of Zhou *et al*. [26]. Mulberroside A accumulation in roots was higher than in shoots of mulberry trees.

Table 3. Contents of mulberroside A in shoots and roots of 4 weeks old mulberry plantlet from *in vitro* culture

Sample	Mulberroside A accumulation (mg/g DW)
Shoot	11.98±0.35[b]
Roots	20.61±0.47[a]

Data represents the mean±standard error. The same letter indicates no statistical differences at $p = 0.05$ according to the Duncan's new multiple range test.

3.4 Effect of MS concentration on growth of mulberry plant in deep-water hydroponic system and mulberroside A accumulation

Since concentraion of plant nutrient, plant growth and bioactive compound production are closely related, different concentrations of MS nutrient solution (1/24, 1/20, 1/16, 1/12 and 1/8) were used in this experiment. After acclimatization, mulberry plants were transferred to a deep-water hydroponic system in glass culture vessels (700 ml) containing 500 ml of different concentrations of MS nutrient solution without sucrose addition. After 4 weeks of cultivation (Figure 2), we found that the growth of mulberry plants in hydroponic culture and mulberroside A content increased with concentration of MS nutrient solution. The highest fresh weight growth index was 2.56 and the highest average shoot length was 34.75 cm in 1/8 MS nutrient solution, results that were significantly difference (Table 4). Moreover, mulberry plants cultivated in 1/8 MS nutrient solution in hydroponic culture gave the highest mulberroside A content in roots (17.58 mg/g DW), as shown in Table 4. The mulberroside A content in shoots was not significantly different in all treatments, and was lower than mulberroside A content in the roots (Table 4). Our results were similar to the results of Zhou *et al*. [26], who found that mulberroside A accumulation in roots was higher than in the shoots of mulberry trees.

Figure 2. A) Cultivation of mulberry trees in deep-water hydroponic system for mulberroside A production from mulberry roots, B) four weeks old mulberry roots harvested for mulberroside A extraction

Table 4. Effects of concentration of MS nutrient solution on growth of mulberry plant and mulberroside A accumulation after 4 weeks of cultivation in hydroponic system

Treatments	Fresh weight growth index	Average shoot length (cm)	Mulberroside A accumulation in shoot (mg/g DW)	Mulberroside A accumulation in root (mg/g DW)
1/24MS	1.17±0.45^{b}	23.20±1.40^{b}	1.74±0.54^{a}	11.68±0.05^{c}
1/20MS	1.20±0.17^{b}	23.63±0.85^{b}	1.82±0.43^{a}	14.79±0.38^{b}
1/16MS	1.23±0.54^{b}	24.60±0.64^{b}	2.12±0.47^{a}	17.13±0.24^{a}
1/12MS	1.41±0.41^{b}	25.50±1.30^{b}	2.13±0.10^{a}	17.46±0.11^{a}
1/8MS	2.56±0.66^{a}	34.75±0.84^{a}	2.34±0.44^{a}	17.58±0.37^{a}

Data represents the mean±standard error. The same letter indicates no statistical differences at $p = 0.05$ according to the Duncan's new multiple range test.

4. Conclusions

According to the findings of this research that was aimed to achieve effective propagation of mulberry plants, the *in vitro* culture of nodal explants to induce multiple shoots in MS medium supplemented with BAP 4 mg/L and NAA 0.5 mg/L could induce 3.33 shoots per explant within 4 weeks. The elongated multiple shoots (1-3 cm) were cut and transferred to different rooting media. The best root development was found on MS medium without PGRs within 10 days. The plantlets with well-developed shoot-roots were transferred to 1/24 MS nutrient solution for 4 weeks acclimatization. The acclimatized plants were transferred to a deep-water hydroponic system in glass vessels containing 500 mL of different concentration of MS nutrient solution without sucrose addition. The results showed that mulberry plants cultivated in 1/8 MS nutrient solution gave the highest growth index. Moreover, this research was intended to study the accumulation of mulberroside A in the shoots and roots of mulberry plant cultured *in vitro* and hydroponic systems. The results showed that mulberroside A accumulation in roots was higher than in the shoots of mulberry plants both *in vitro* culture and hydroponic system. The hydroponic system can be used

for producing root biomass containing bioactive compounds [19- 24]. In order to optimize mulberroside A productivity, the future work should be aimed at producing root biomass in hydroponic culture with the combination of a range of biotic factors such as elicitors, plant hormones, and precursors that are able to induce mulberroside A accumulation in roots.

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