Effect of Elicitors on Mulberroside A Production in *Morus alba* Root Cultures

การศึกษาผลของสารกระตุ้นต่อการสร้างสารมัลเบอร์โรไซด์เอในรากเพาะเลี้ยงต้นหม่อน

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ABSTRACT

Root bark of *Morus alba* has been used as traditional medicine for anti-tussive, anti-inflammation and anti-asthmatic. Besides that *M. alba* extracts are also widely uses in natural health product. In this study, mulberroside A (MuA), major bioactive compound was produced by tissue culture technique with elicitation by elicitors. To investigate elicitation effects on mulberroside A production in root cultures, enzyme-linked immunosorbent assay were used to determine the MuA. The results show effect of elicitors including yeast extract 2 mg/ml, methyl jasmonate 200 µM, salicylic acid 100 µM, 3% v/v *Phoma* sp. and 3% v/v *Bacillus subtilis* extract significantly exhibited enhancement of MuA production in *M. alba* root cultures (556.32%, 69.89%, 20.29%, 26.29% and 37.52% increase respectively)

บทคัดย่อ

เปลือกรากหม่อนจัดเป็นยาแผนโบราณซึ่งถูกนำมาใช้เพื่อการรักษาอาการไอ อักเสบ และหอบหืด นอกจากนี้ปัจจุบันมีการนำสารสกัดจากหม่อนมาใช้ในผลิตภัณฑ์สุขภาพทางธรรมชาติอย่างกว้างขวาง ในการศึกษาครั้งนี้สารมัลเบอร์โรไซด์เอซึ่งเป็นสารสำคัญในการออกฤทธิ์ทางชีวภาพถูกผลิตขึ้นโดยใช้เทคนิคเพาะเลี้ยงเนื้อเยื่อร่วมกับการเติมสารกระตุ้น เพื่อศึกษาผลของสารกระตุ้นต่อการสร้างสารมัลเบอร์โรไซด์เอโดยใช้เทคนิคอีไลซ่า ผลการศึกษาพบว่าการเติมสารสกัดจากยีสต์ 2 mg/ml, เมทิลจัสโมเนต 200 µM, กรดซาลิกซิลลิก 100 µM, สารสกัดจากเชื้อรา *Phoma* sp. 3% v/v และสารสกัดจากแบคทีเรีย *Bacillus subtilis* extract 3% v/v สามารถเพิ่มการผลิตสารสารมัลเบอร์โรไซด์เอในรากเพาะเลี้ยงต้นหม่อนได้ (เพิ่มขึ้น 556.32%, 69.89%, 20.29%, 26.29% และ 37.52% ตามลำดับ)

Key Words: *Morus alba,* Mulberroside A, Elicitor

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Introduction

Root bark of *Morus alba* (sang bai pi or cortex mori) was used as traditional medicine for anti-tussive, anti-inflammation and anti-asthmatic (Piao et al., 2010). Mulberroside A (MuA, Figure 1) is a major stilbene glycoside from root bark of mulberry, has been identified as the active compound. MuA can be converted by microbial intestine or directly by glycosidase to aglycone part, oxyresveratrol (Kim et al., 2010; Mei et al., 2011). Oxyresveratrol shows many pharmacological effects including anti-tyrosinase activity (Tengamnuay et al., 2006), anti-viral activity (Galindo et al., 2011; Lipipun et al., 2011), antioxidant activity (Aftab et al., 2010), hepatoprotective effect (Shi et al., 2008) and neuroprotective effect (Horn et al., 2004). To date, mulberry root extracts are widely uses in natural health products and cosmetic in the market.

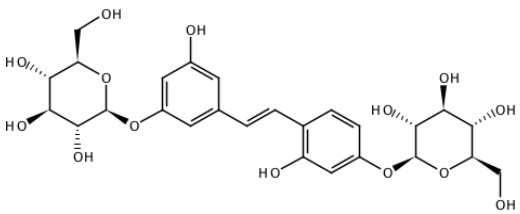

Fig. 1 mulberroside A (MuA) structure

Tissue cultured can be growth in laboratory and accumulating high amount of chemicals found in parent plant with short growth cycles (Rao and Ravishankar, 2002). Stimulation of plant defense response by using elicitors were reported for enchants plant secondary metabolites. Therefore, tissue culture with elicitation method may be effective tool to solve problem related to production of secondary metabolites from natural plants including environmental factors, political and labor instability (Smetanska, 2008). Salicylic acid, methyl jasmonate, yeast extract, chitosan and fungal extract were reported as stilbenes elicitiors (Aziz et al., 2006; Belhadj et al., 2006; Roat and Ramawat, 2009). Besides that endophytes and common microbial isolated from their host plant have been reported for elicitation effect on production of their host bioactive metabolites (Chong et al., 2009; Orlita et al., 2007). Therefore, this study presents effect of well-known elicitors and microbial isolated from *M. alba* on MuA production in *M. alba* root culture.

Objectives of the study

1. To determine MuA production from *M. alba* root culture.
2. To study elicitation effects from elicitors on MuA production in *M. alba* root culture.

Methodology

Plant materials and sample preparation

M. alba samples were collected from faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand (April 2011).

Young twigs of *M. alba* were rinsed with tap water, then sterilized with 20 and 10% sodium hypochlorite for 15 minutes, respectively. After that the explants were washed with sterilized distill water several times and plated on MS medium. Root culture was obtained from root part of the *M. alba* cultures on half strength MS liquid medium supplemented with 1 mg/l NAA.

Dried powdered of plant samples (10 mg) were weighted, extracted with methanol 500 µl and then sonicated for 15 min. The extracts were centrifuged at 3,000 rpm for 3 min to collect the supernatant. This extraction procedure was repeated four times. The combined extract were evaporated at 50°C and re-

dissolved in methanol 1 ml. Consequently, sample solutions were diluted into appropriated concentration for MuA determination by ELISA

Indirect competitive ELISA

After coating 96-well immunoplate with MuA-OVA, it was then treated with of PBS containing 1% of gelatin to reduce non-specific adsorption. Various concentrations of MuA or samples (50 µl) dissolved with 20% methanol were incubated with 50 µl of PAb solution for 1 h. The plate was washed three times with TPBS, and then incubated with 100 µl of 1,000 fold dilution of peroxidase-labeled anti-rabbit IgG solution for 1 h. After washing the plate three times with TPBS, 100 µl of substrate solution was added to each well and incubated for 15 min. The absorbance was measured by a micro plate reader at 405 nm

Elicitors preparation

M. alba explants were rinsed with tap water, dipped in 2% sodium hypochlorite (10 min), 70% for 30 seconds and washed with sterilized distill water several times. Surfaces of explants were removed by cutting under sterile conditions. Small piece of surface sterilized explants were plated on potato dextrose (PDB) and Lysogeny broth (LB). Difference characteristic microorganism were picked and subcultured at least 3 times to obtained single strain. Isolated microorganisms were transferred onto liquid potato dextrose (PDB) or Lysogeny broth (LB). Seven days old microbial were harvested, and then centrifuged at 10,000 rpm. Supernatant were removed and replaced with distilled water. Microbial suspension solutions were autoclaved and centrifuged at 10,000 rpm. Sterilized supernatants were collected and stored at 4°C until used as elicitors.

Selected concentration of methyl jasmonate (200 µM), yeast extract (2 mg/ ml), chitosan (10 mg/l) and salicylic acid (100 µM) also prepared by filtration or sterilization by autoclaved. Sterilized elicitors were added to 20 days old root culture. Root culture samples were harvested on day 21, dried at 50 °C for 2 days, then extract with the same method as plant samples.

Results

Study for the growth pattern of *M. alba* root culture was started with 250 ml flasks containing 30 ml medium. Every week, 3 flasks of root cultures were harvested. As show in Fig. 2, dry weight (DW) and intracellular MuA concentration of root cultures significantly increase in first 3 weeks (10.83±2.23, 14.38±0.75 and 18.47±2.11 mg/g DW, respectively) then slow down and decrease after 4 weeks (18.68±3.89, 12.79±2.90 and 15.02±1.56 mg/g DW respectively). Extracellular MuA concentration slightly increases in 2 weeks, then slowdown. When compared with intracellular MuA level, extracellular MuA centent is low (3.0-7.3 folds approximately), with these reasons 20 days old root cultures were chosen for elicitation study.

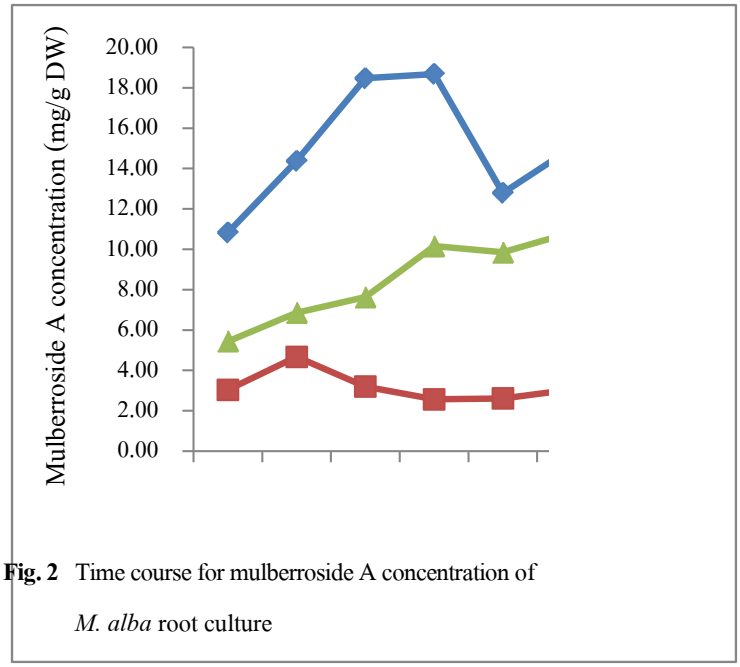

Fig. 2 Time course for mulberroside A concentration of *M. alba* root culture

Microbial were obtained from sterilized surface twig and fibrous root of *M. alba.* Observable 11 differences strains were isolated, prepared and used as elicitors in pilot study. 2 of 11 strains show elicitation effect on mulberroside A production. Effective strains were identified as *Phoma* sp. and *Bacillus subtilis*.

Table 1 Effect of elicitors on MuA production

Sample		MuA accumulation (mg/g DW)	
		Intracellular	Extracellular
Root cultures	+ Yeast extract	17.12±0.20	6.69±0.08
	+Methyl jasmonate	4.78±0.75	1.38±0.22
	+Chitosan	3.38±0.67	1.02±0.20
	+Salicylic acid	3.03±0.25	1.34±0.11
	+*Trichoderma* sp.	3.41±0.46	1.19±0.16
	+*Phoma* sp.	3.87±0.46	0.71±0.08
	+*B. subtilis*	3.69±0.18	1.30±0.06
	Control	2.98±0.26	0.65±0.06
Intact plant	Fibrous root	5.32±0.37	-
	Root	22.39±1.06	-
	Root bark	26.86±2.69	-

As shown in table 1, addition of yeast extract at 2 mg/ml exhibited the highest elicitation effect on total MuA production (intracellular and extracellular concentration), 556.32% compared with control (23.81±0.3 and 3.63±0.32 mg/g DW, respectively). The concentration of MuA in root culture elicitation by yeast extract not only higher than that found in intact fibrous root, but also similar level as MuA from root and root bark of several years old intact mulberry. Methyl jasmonate 200 µM, salicylic acid 100 µM, 3% v/v *Phoma* sp. and 3% v/v *Bacillus subtilis* extract significantly increase MuA production (69.89%, 20.29%, 26.29% and 37.52% increase respectively), while 1% v/v *Trichoderma* sp. extract and 10 mg/l chitosan did not significantly increase mulberroside A production.

Discussion and Conclusions

Various elicitors were added to root culture in highly growth and high MuA production period. The result shows positive elicitation effects of microbial isolate from host in the same way with previous report (Orlita et al., 2008; Wang et al., 2001).

This study did not focus on the best condition for MuA production. Optimization of various factor such as concentration or using combination of elicitors, inoculums density, nutrient content and light condition possible to increase MuA yield in root culture. However, the result shows that 3 weeks old of *M. alba* root culture with yeast extract as elicitor produced similar yield of MuA to several years old intact *M. alba* root and root bark (23.81±0.3, 22.39±1.06 and 26.86±2.69 mg/g DW, respectively). For these reasons we conclude that root culture of *M. alba* with elicitation treatment may be interesting method for production of MuA in large scale.

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