CHAPTER IV
RESULTS AND DISCUSSION

1. Production and Characterization of Polyclonal Antibody Against MuA

1.1 The MALDI-TOF-MS spectrum of hapten-carrier protein conjugates

The MALDI-TOF-MS spectrum of MuA-BSA conjugate appeared approximately at m/z 80,444 (Figure 7). According to molecular weights of BSA and MuA are 66,382 and 508 respectively, hapten number of MuA-BSA is approximately 25. In general, 8–25 molecules of hapten conjugated with carrier protein in the conjugate were sufficient for immunization (Putalun et al., 2002).

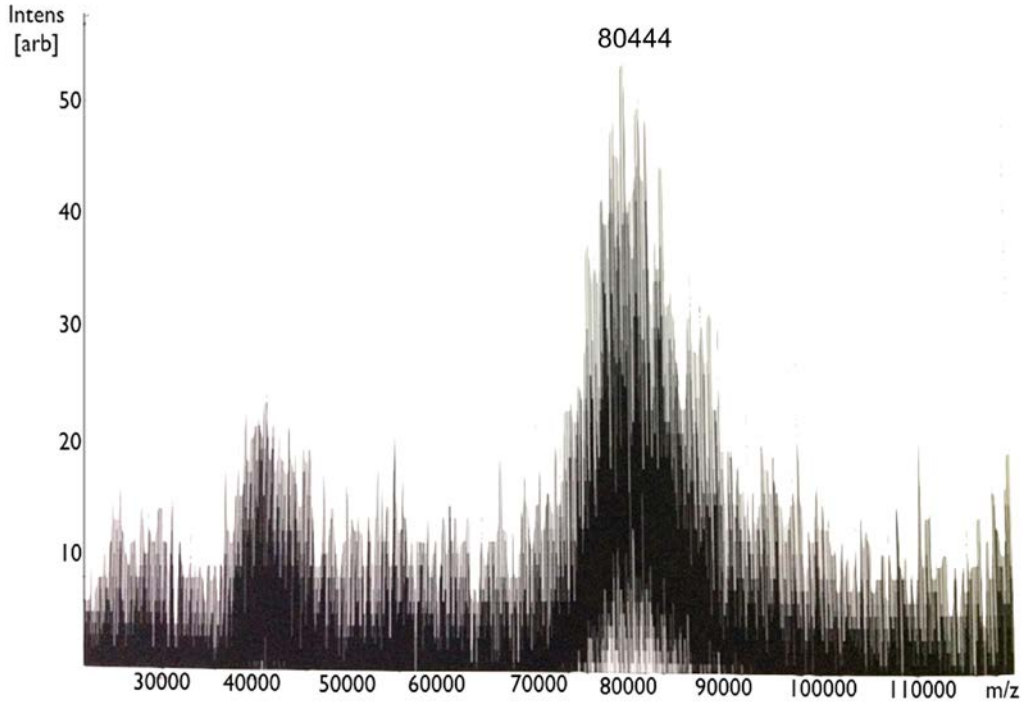

Figure 7 The MALDI-TOF mass spectrum of the MuA–BSA conjugate

1.2 Characterization of polyclonal antibody against MuA

After the third immunization, the rabbit serum was applied to a protein G column. The rabbit IgG was obtained and examined the reactivity of the antibody. Various concentrations of anti-MuA PAb were added and determined by indirect ELISA. The result shows reactivities of PAb against MuA (Figure 8). The absorbance of antibody at 2 μg/ml is approximately 1.0. Therefore, this concentration was chosen for indirect competitive ELISA.

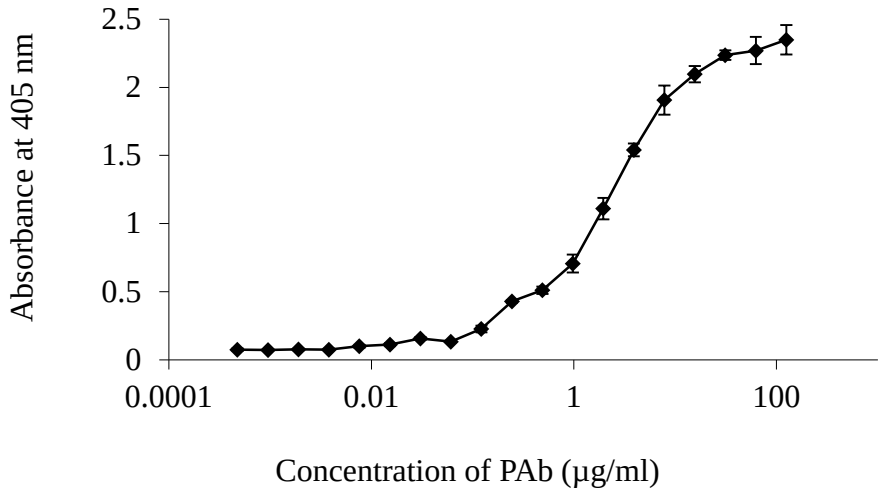

Figure 8 Reactivities of PAb against MuA

1.3 Method validation

Sensitivity of the method was validated by measuring concentration range of MuA. The optimal concentration range of assay extends from 0.17-15.62 μg/ml (Figure 9). The concentrations 0.39, 0.78, 1.56, 3.13, 6.25 and 12.50 μg/ml were selected for indirect competitive ELISA. The sensitivity of this ELISA method is slightly higher than reported HPLC method, which measure ranged from 0.8-500 μg/ml (Piao et al., 2010).

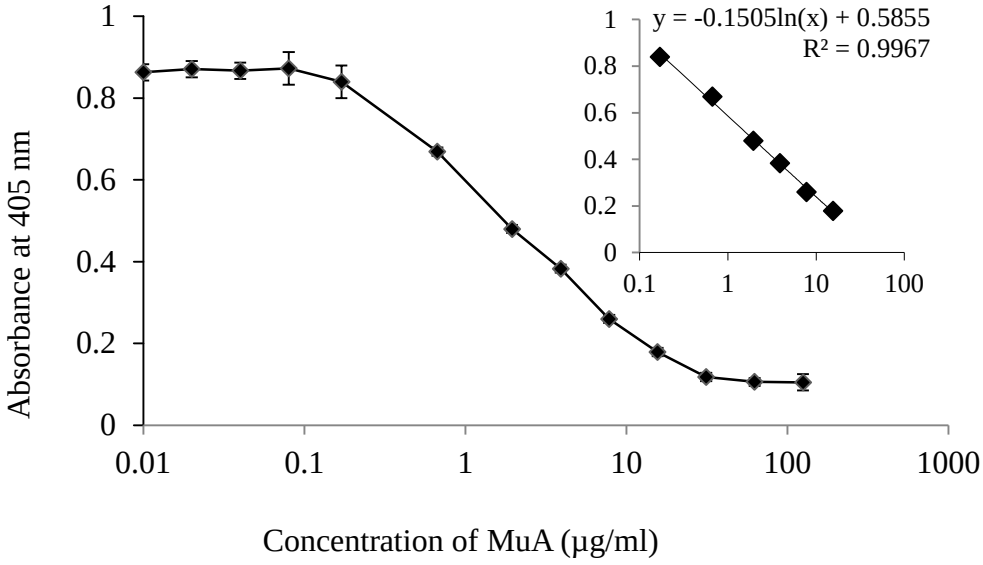

Figure 9 Standard ELISA curve for the determination of MuA

The specificity of the assay was evaluated by examined cross-reactivities of PAb with indirect competitive ELISA and calculated using method of Weiler & Zenk (1976). Table 2 shows that anti-MuA PAb did not found any cross-react with structure related compounds including oxyresveratrol, aglycone part of MuA. These result suggests that the anti-MuA PAb is highly specific for MuA. These results indicate that the sugar parts are essential for reactivity and specificity of the anti-MuA PAb. Therefore, the ELISA method is specific for determination of MuA.

Table 2 Cross reactivities of anti-MuA polyclonal antibody

Compound	Classification	Cross-reactivity (%)
MuA	Stilbenoids	100
Oxyresveratrol		<0.01
Resveratrol		<0.01
Moscatilin		<0.01
Artocarpin	Flavonoids	<0.01
Morusin		<0.01

Table 2 Cross reactivities of anti-MuA polyclonal antibody (Cont.)

Compound	Classification	Cross-reactivity (%)
Norartocarpetin	Flavonoids	<0.01
Norcycloartocarpin		<0.01
Isocyclomorusin		<0.01
Rutin		<0.01
Cycloartobiloxanthone		<0.01
Glycyrrhizin	Terpenoids	<0.01
Artemisinin		<0.01
TDZ	Cytokinins	<0.01
NAA	Auxins	<0.01

Table 3 Intra- and inter-assay precisions of MuA analysis by ELISA using PAb against MuA (n=5)

MuA concentration (μg/ml)	Intra-assay RSD (%)	Inter-assay RSD (%)
0.20	3.01	3.14
0.39	2.07	3.34
0.78	3.11	2.79
1.56	2.88	3.02
3.13	3.31	4.71
6.25	4.58	4.85
12.5	4.92	4.84

Intra-assay and inter-assay precisions were determined. Table 3 shows that the maximum RSD of intra- and inter-assay were 4.92 % and 4.85%, respectively. The factors that affect variations of the ELISA method might be due to temperature during

incubation and variation in preparation reagents. Therefore, a standard curve should be generated in every assay to reduce variations.

Recovery experiment was studied to evaluate the accuracy. After spiking solutions of various concentrations of MuA into *M. alba* sample, the sample extracts were prepared, and the content of MuA in each sample was determined by the developed ELISA method. As shown in Table 4, the percentages of recovery in the range of 97.6–101.4% with a maximum RSD of 4.13%, indicated high extractability and recovery obtained from developed method.

Table 4 Recovery of MuA determined by ELISA (n=3)

Concentration of MuA added (µg/ml)	MuA content (µg/ml)	Recovery (%)	RSD (%)
0	206.41±7.36	-	3.56
18.75	228.27±9.42	101.4	4.13
37.50	242.46±3.87	99.4	1.59
75	283.07±10.07	100.3	3.59
150	352.95±10.28	97.6	3.96

In order to determine the correlation between the ELISA and HPLC results, various samples of *M. alba* were determined using the developed method. Table 5 shows the results from MuA contents in different *M. alba* samples. The concentrations of MuA determined by ELISA had a good correlation with those determined by HPLC. The correlation between the results from the ELISA and HPLC has a coefficient of determination (R^2) of 0.994 (Figure 10). The root bark of *M. alba* showed the highest amount of MuA (26.86±2.69 mg/g DW), followed by root and fibrous root, respectively (Table 5). MuA was detected in the twig of *M. alba* sample from Khon Kaen using ELISA method whereas HPLC method could not detect due to lower than limit of detection. MuA content in root and cell suspension cultures of *M. alba* were 19.34±0.53 and 10.31±0.76 mg/g DW, respectively. This result confirms that the developed ELISA method is significantly more sensitive than the HPLC

method. In addition, MuA was not found in leaf of *M. alba* using both assays suggesting that the variation of MuA accumulation in different part of plant. From these results, the developed ELISA method was applicable for detection of MuA in plant samples and *in vitro* cultures of *M. alba*. The prior advantages of the developed ELISA system over traditional analytical methods are its simplicity, rapidity and sensitivity. Therefore, this validated method is reliable for MuA determination in various MuA containing plant materials and final product.

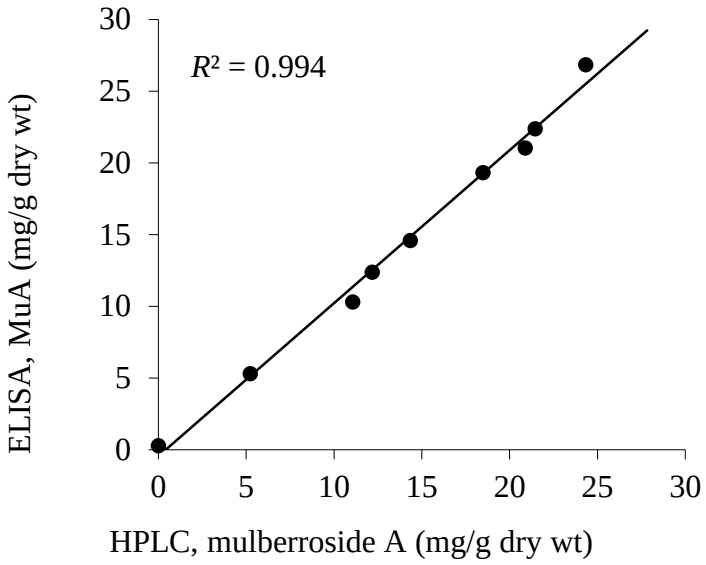

Figure 10 Correlation between MuA from ELISA and HPLC method

Table 5 Determination of MuA in *M. alba* using ELISA and HPLC

M. alba sample	Concentration of MuA (mg/g DW)	
	HPLC	ELISA
Khon Kaen, Thailand		
Leaf	ND	ND
Twig	ND	0.29±0.10
Fibrous root	5.24±0.39	5.32±0.37
Root	21.44±1.69	22.39±1.06

Table 5 Determination of MuA in *M. alba* using ELISA and HPLC (Cont.)

M. alba sample	Concentration of MuA (mg/g DW)	
	HPLC	ELISA
Khon Kaen, Thailand		
Root bark	24.32±0.20	26.86±2.69
Fukuoka, Japan		
Leaf	ND	ND
Twig	ND	ND
Fibrous root	12.17±0.16	12.40±1.40
Root	14.34±1.14	14.60±0.85
Root bark	20.88±0.76	21.05±0.38
In vitro culture		
Cell suspension culture	11.06±0.32	10.31±0.76
Root culture	18.47±2.11	19.34±0.53

ND = not detected

2. The Production of MuA from *In vitro* Cultures of *M. alba*

2.1 Growth pattern of *M. alba* cell cultures

The callus cultures of *M. alba* was obtained after the induction on MS medium supplemented with 0.1 mg/l TDZ and 1 mg/l NAA. The regenerated shoots and leaves can be observed from the leaf-derived callus (Figure 11A).

The leaf-derived calli were subcultured on the same solid medium (Figure 11B) several times before transferred into liquid MS medium supplemented with 0.1 mg/l TDZ and 1 mg/l NAA (Figure 11C) to obtain cell suspension cultures. After transferring, releasing of secondary metabolites in the liquid medium of cell suspension was observed. The cultured medium was changed to more yellow in a few days (Figure 11D). For this reason, cultured medium of *M. alba* were also investigated MuA accumulation.

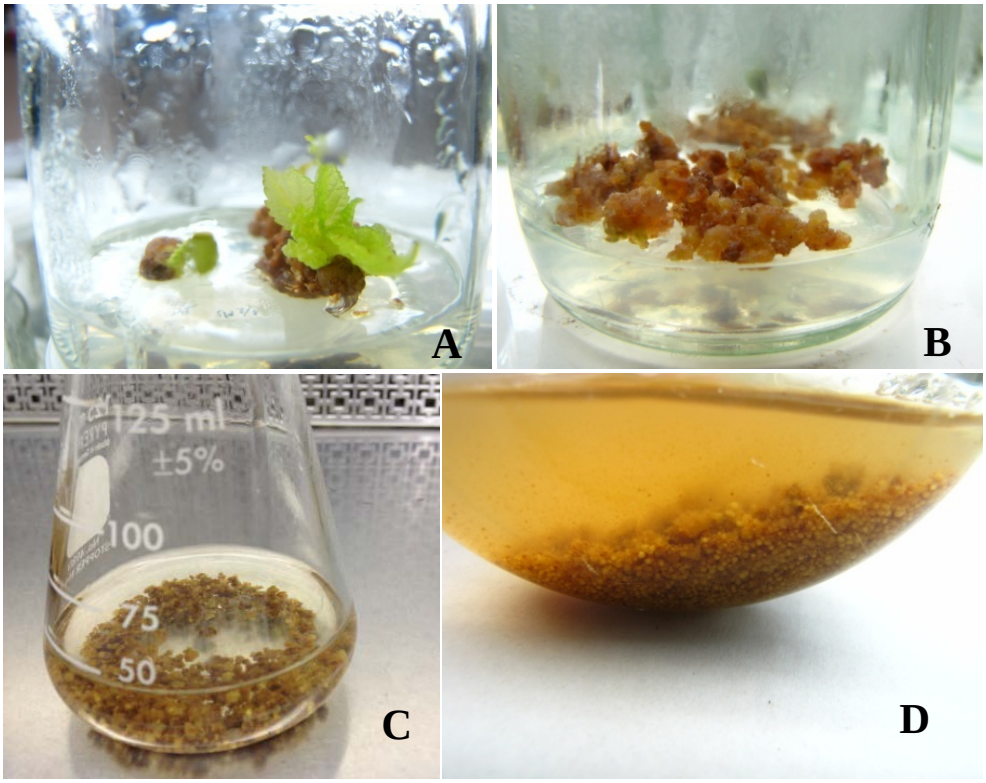

Figure 11 Leaf-derived callus of *M. alba*, (A) and (B) callus cultures, (C) and (D) cell suspension in MS medium with 0.1 mg/l TDZ and 1 mg/l NAA supplements

The growth pattern of *M. alba* cell cultures study were started with 250 ml flask containing 30 ml medium. Every week, cell cultures and medium were harvested to investigate biomass production and MuA concentration. The results show biomass of cell cultures were increased during first four weeks, then slow down and go on stationary phase at the fifth week (Figure 12). Intracellular MuA concentration was slightly decreased in second week then the concentrations were increased until fourth week. After that the intracellular MuA accumulations were slow down and decreased. Extracellular MuA accumulation was also slightly decreased at second week and then increased at third week follow with slow down and decreased after 4 weeks (Table 6). The total MuA concentrations were rapidly increased during 2-4 weeks (Figure 12). The high biomass and MuA production period are during 3-4 weeks. Therefore,

twenty-one days old of cell cultures were selected as harvested time for the elicitation treatment

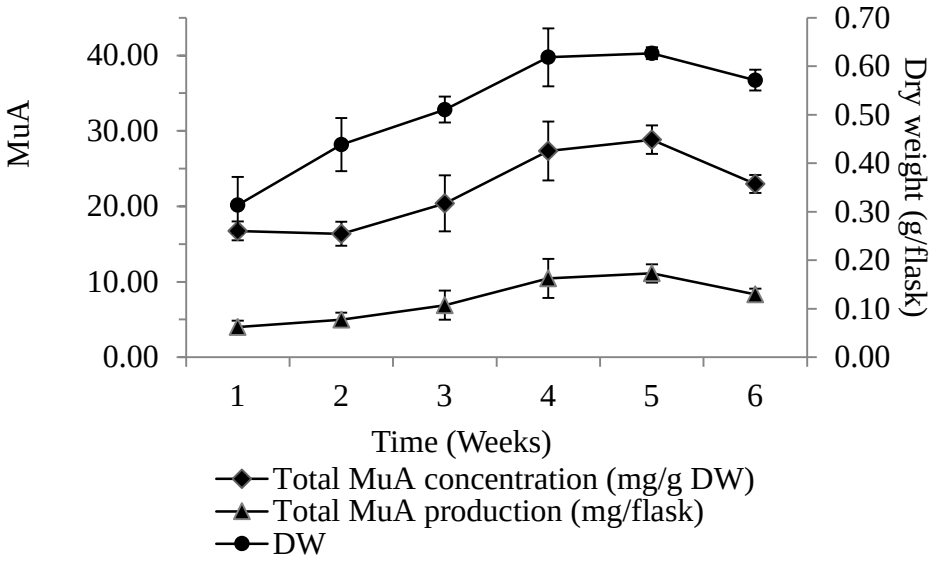

Figure 12 Time course for total MuA contents of *M. alba* cell suspension (n=3)

Table 6 Time course for intracellular and extracellular MuA concentration of *M. alba* cell cultures (n=3)

Time	MuA concentration (mg/g DW)			Biomass
(weeks)	Intracellular	Extracellular	Total	(g DW/flask)
1	11.50 ± 1.13	1.24 ± 0.12	12.74 ± 1.25	0.31 ± 0.06
2	10.45 ± 1.53	0.93 ± 0.07	11.38 ± 1.60	0.44 ± 0.05
3	12.14 ± 3.56	1.36 ± 0.17	13.50 ± 3.73	0.51 ± 0.03
4	15.70 ± 3.68	1.19 ± 0.21	16.89 ± 3.89	0.62 ± 0.06
5	16.72 ± 1.82	1.00 ± 0.06	17.72 ± 1.88	0.63 ± 0.01
6	12.88 ± 1.14	1.73 ± 0.06	14.61 ± 1.20	0.57 ± 0.02

2.2 Growth pattern of *M. alba* root cultures

Root cultures of *M. alba* were regenerated from cell cultures on MS medium supplemented with 1 mg/l NAA (Figure 13A), then the regenerated roots were transferred into liquid MS medium supplemented with 1 mg/l NAA (Figure 13B). Growth rates and MuA productions both intra- and extracellular of root cultures were investigated.

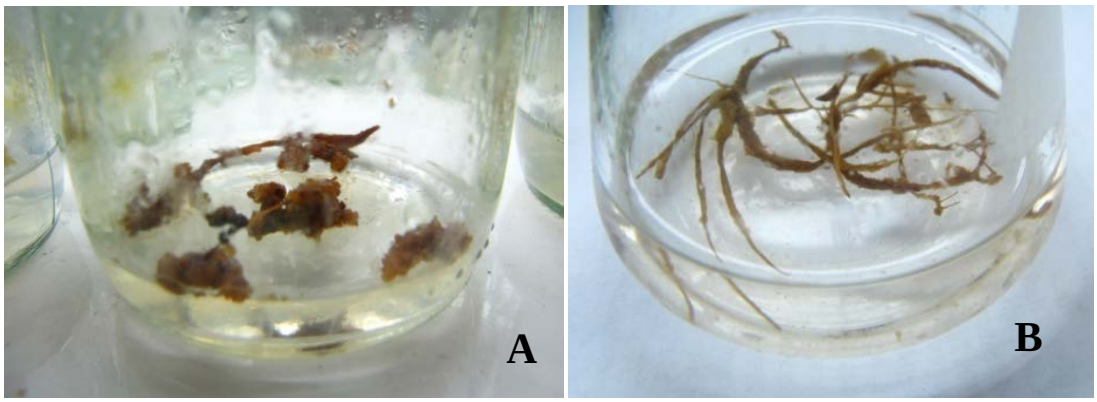

Figure 13 Regenerated root (A) and *in vitro* root cultures of *M. alba* in MS medium supplemented with 1 mg/l NAA (B)

Table 7 Time course for intracellular and extracellular MuA concentration of *M. alba* root cultures (n=3)

Time (weeks)	MuA concentration (mg/g DW)			Biomass (g DW/flask)
	Intracellular	Extracellular	Total	
1	10.83 ± 2.33	3.04 ± 0.60	13.87 ± 2.93	0.11 ± 0.00
2	14.38 ± 0.75	4.67 ± 0.59	19.05 ± 1.34	0.14 ± 0.04
3	18.47 ± 2.11	3.19 ± 0.58	21.66 ± 2.69	0.15 ± 0.03
4	18.68 ± 3.89	2.57 ± 0.41	21.25 ± 4.30	0.20 ± 0.02
5	12.79 ± 2.90	2.61 ± 0.31	15.40 ± 3.21	0.20 ± 0.04
6	15.02 ± 1.56	3.06 ± 0.39	18.08 ± 1.95	0.22 ± 0.03

M. alba root cultures were also started with 250 ml flasks containing 30 ml medium. Root cultures were harvested every week (n=3). Figure 14 and Table 7 show dry weight and intracellular MuA concentration of root cultures which highly increased in first three weeks. The extracellular MuA concentrations slightly increased in two weeks of cultures then slow down. The intracellular MuA level is approximately 3.0-7.3 folds higher than that found in extracellular MuA. Therefore, three weeks old root cultures were also chosen for the elicitation study.

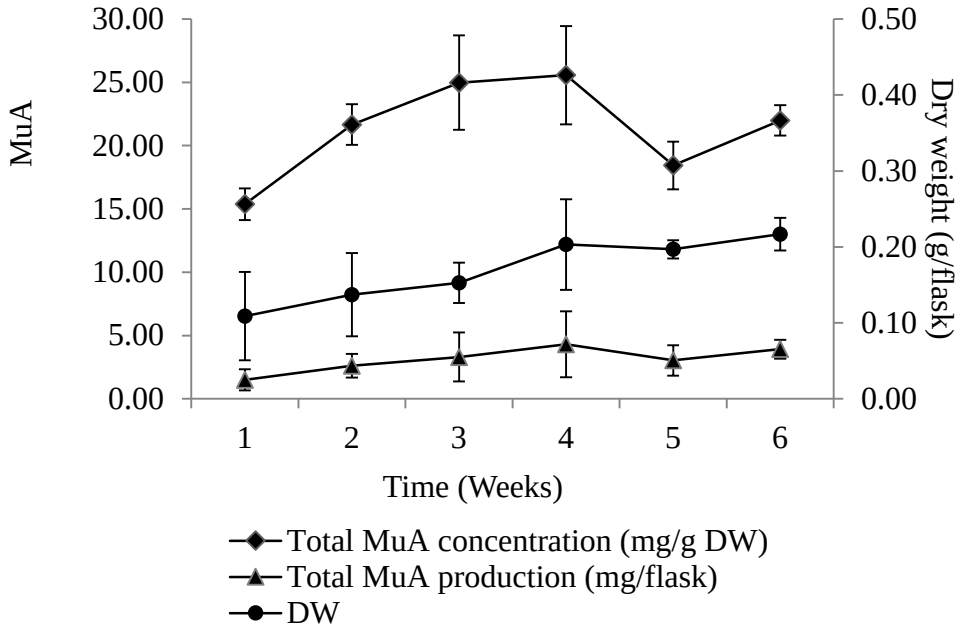

Figure 14 Time course for total MuA contents of *M. alba* root cultures (n=3)

2.3 Effects of elicitors on MuA accumulation in cell suspension cultures of *M. alba*

Effects of well-known and *M. alba*-associated microbial elicitors on the MuA accumulation cell suspension cultures were studied by addition various concentration and exposure time of each elicitor. The MuA concentrations were determined by indirect competitive ELISA.

2.3.1 Yeast extract

The different concentrations of yeast extract (YE) (0.5, 1 and 2 mg/ml) were added on 18, 19 and 20 days old cell suspension cultures of *M. alba*. In YE

treated groups with 24 h exposure time, it was significantly dose dependent enhanced both intracellular and total MuA levels by the addition all of selected concentration of the YE and the highest total MuA level was obtained by the addition of 2 mg/ml YE (3.24 folds). In contrast, 48 h after addition various concentrations of YE, total MuA level was significantly enhanced only in the cell cultures treated with 1 mg/ml YE (2.02 folds). After 72 h exposure time, the cell treated with 0.5 and 1 mg/ml were significantly enhanced the MuA levels (1.81 and 2.13 folds, respectively, Figure 15). These results show significantly positive elicitation effects of YE on intracellular MuA accumulation in the cell cultures but extracellular MuA levels in the cell cultures were significantly decreased at all YE treatment groups (average 0.61 folds) compared with control group and no significant difference of MuA level within treatment groups (Figure 15 and Table 8).

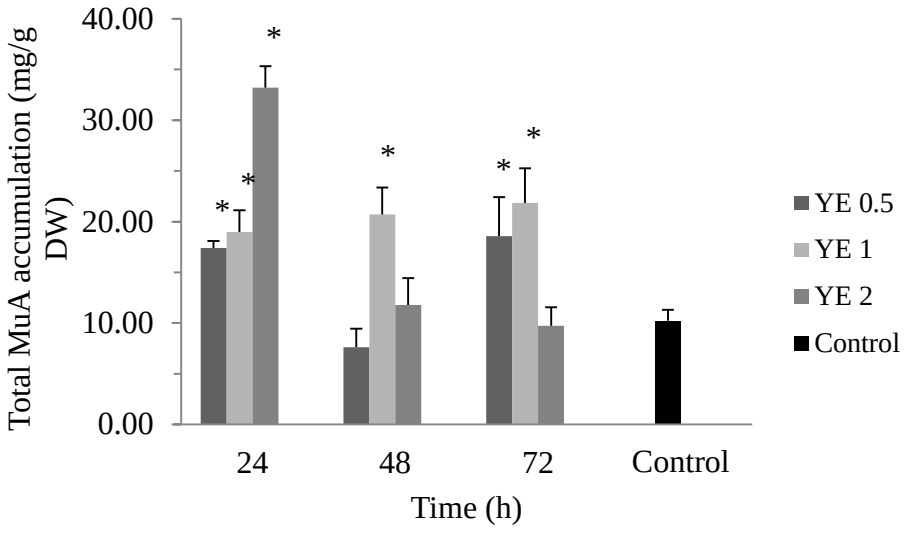

Figure 15 Effects of yeast extract on total MuA accumulations in cell cultures of *M. alba* (n=3, * indicates significantly different from control group at the $P<0.01$)

2.3.2 Methyl jasmonate

Cell suspension cultures of *M. alba* were treated by 50, 100 and 200 µM MJ on days 18, 19 or 20. Total and intracellular MuA levels in the cell cultures were significantly increased at all concentrations and exposure time (Figure 16 and Table 8). The highest total MuA levels was obtained in 200 µM MJ with 48 h exposure time

group (2.31 folds). However, most extracellular MuA concentrations in MJ treated group are significantly lower than control (average 0.77 folds), except 50 µM exposure time and 200 µM with 48 h groups that are slightly higher extracellular MuA concentrations with no significantly difference (Table 8).

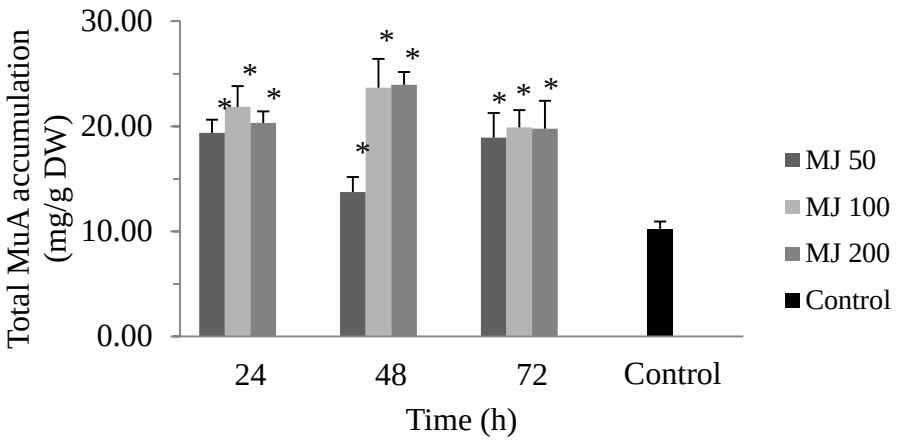

Figure 16 Effects of methyl jasmonate on total MuA accumulations in cell cultures of *M. alba* (n=3,* indicates significantly different from control group at the $P<0.01$)

2.3.3 Chitosan

Cell cultures of *M. alba* were treated with 10, 20 and 50 mg/l of chitosan (CT) at days 18, 19 and 20. Figure 17 shows that total MuA contents did not significantly enhanced by the addition with CT. Contrary, the total MuA levels were significantly decreased in 50 mg/l CT treated with 48 h exposure time and 10 mg/l CT treated with 72 h exposure time groups (0.78 and 0.60 folds, respectively, Figure 17). The result shows no significantly enhancement of intracellular MuA was occurred by the treatment but extracellular MuA was significantly increased in 10 mg/l CT treated group (Table 8).

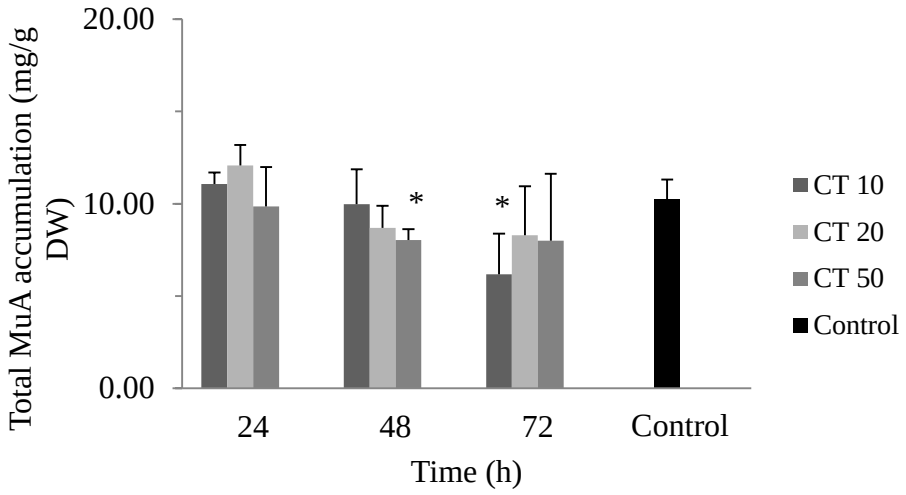

Figure 17 Effects of chitosan on total MuA accumulations in cell cultures of *M. alba* (n=3,* indicates significantly different from control group at the $P<0.01$)

2.3.4 Salicylic acid

Cell cultures of *M. alba* were also treated with 50, 100 and 100 µM salicylic acid (SA) on days 18, 19 and 20. Total MuA levels were significantly increased in almost concentrations and durations in SA treatment except 200 µM SA with 48 exposure time group. The result show total MuA levels were high in 100 and 200 µM SA with 24 h exposure groups (3.71 and 3.04 folds, respectively, Figure 18). The result show the dose dependent elicitation effects of SA treatments with 24 h exposure time on the extracellular MuA production. The extracellular MuA levels in 100 and 200 µM SA with 24 h exposure time groups are significantly higher than control group (2.94 and 3.03 folds, respectively). Whereas, the extracellular MuA were no significantly effected in 50 µM SA with 24 h exposure time and in all concentrations of SA with 48 h groups. The extracellular MuA contents were significantly decreased in all SA treated with 72 h exposure time groups (average 0.49 folds), compared with the control (Table 8).

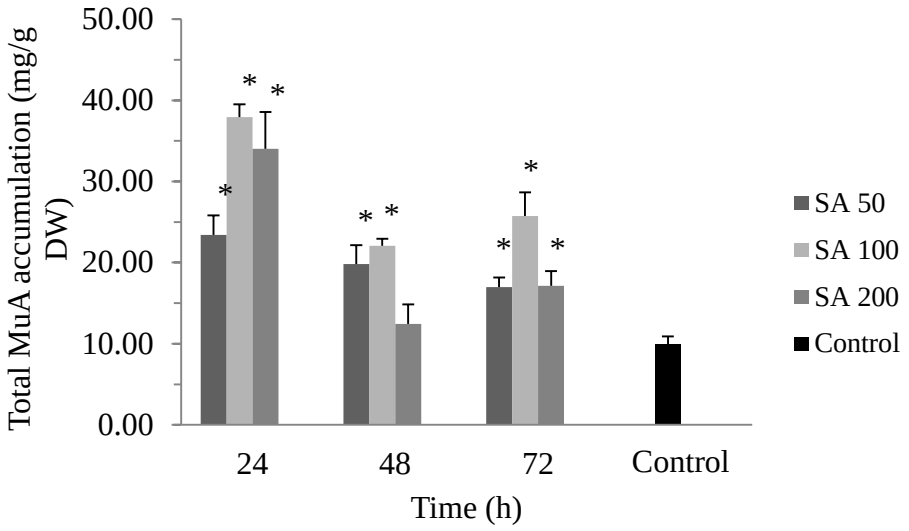

Figure 18 Effects of salicylic acid on total MuA accumulations in cell cultures of *M. alba* (n=3,* indicates significantly different from control group at the $P<0.01$)

2.3.5 *M. alba*-associated microbial elicitors

To investigate the effect of *M. alba*-associated microbial elicitors on MuA production, 1, 2 and 3% v/v extracts of microbial isolated from *M. alba* were added on 18, 19 and 20 days old cell cultures. Three extracts of the microorganisms were indicated as positive elicitation effect strains. The microbial were identified as *Phoma* sp., *Bacillus subtillis* and *Trichoderma* sp.

2.3.5.1 *Phoma* sp. extract

The total MuA accumulation was significantly increased after treated with 3% v/v *Phoma* sp. extracts (PE) with 24 h exposure time (1.54 folds, Figure 19). Table 8 shows the intracellular MuA levels were no significantly difference between PE treatments and control groups. Extracellular MuA levels were significantly increased in 3% v/v PE treated with 24 h exposure time and in 1% and 3% v/v PE treated with 48 h exposure time groups (2.84, 1.48 and 3.64 folds, respectively).

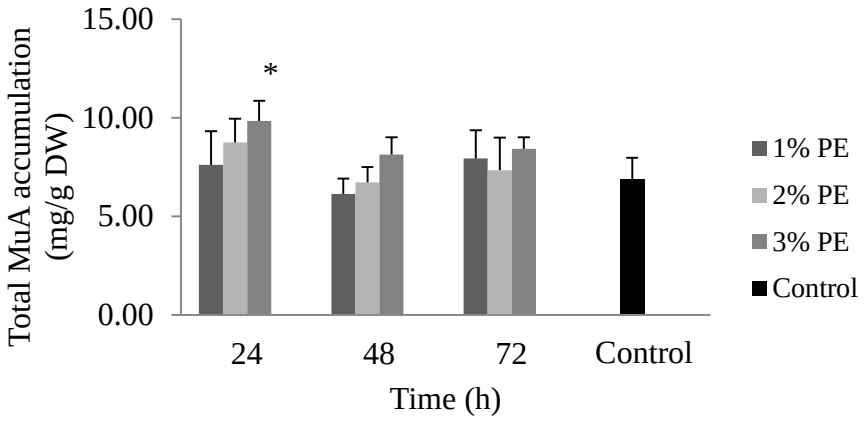

Figure 19 Effects of *Phoma* sp. extracts on total MuA accumulations in cell cultures of *M. alba* (n=3,* indicates significantly different from control group at the $P<0.01$)

2.3.5.2 *Bacillus subtillis* extract

The total MuA levels were significantly increased in 3% v/v treated *B. subtillis* extract (BE) with 24 exposure time group (1.78 folds), 1% BE treated with 48 h exposure time group (1.39 folds), 1 and 2% v/v BE treated with 72 h exposure time groups (2.16 and 2.06 folds, respectively), compared with control (Figure 20). Similar to intracellular MuA levels which the MuA were increased in the same groups. While, extracellular MuA levels were increased in 2 and 3% v/v BE treated with 24 h exposure time group and 1% v/v BE treated with 72 h exposure time group (1.32, 2.24 and 2.53 folds, respectively).

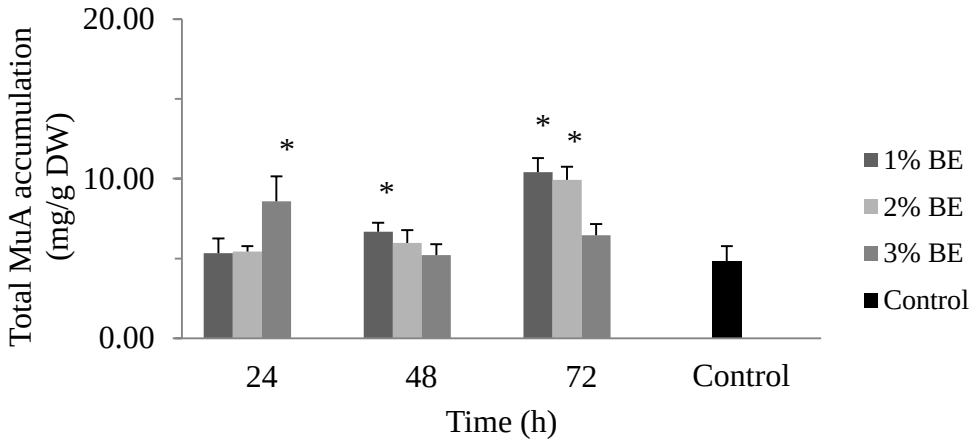

Figure 20 Effects of *B. subtillis* extracts on total MuA accumulations in cell cultures of *M. alba* (n=3,* indicates significantly different from control group at the $P<0.01$)

2.3.5.3 *Trichoderma* sp. extract

The total MuA levels were significantly increased in all concentration treated *Trichoderma* sp. extract (TE) with 24 exposure time groups (1.35 folds, Figure 21), in the same way with intracellular MuA levels. Whereas, the extracellular MuA levels were significantly increased in all TE treatments (6.69 folds). The extracellular MuA levels were dose dependence increased at 24 h exposure time and the highest extracellular MuA level was archieved in 2% TE treated group (9.61 folds, Table 8).

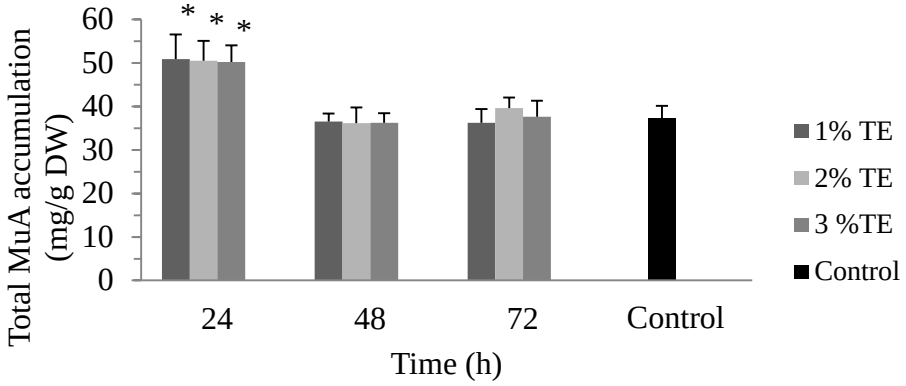

Figure 21 Effects of treated *Trichoderma* sp. extracts on total MuA accumulations in cell cultures of *M. alba* (n=3,* indicates significantly different from control group at the $P<0.01$)

Table 8 Effects of elicitors on MuA accumulations in cell cultures of *M. alba* (n=3,* indicates significantly different from control group at the $P<0.01$)

Elicitor	Exposure time (h)	Dose	MuA accumulation (mg/g DW)		
			Intracellular	Extracellular	Total
YE	24	0.5 mg/ml	17.18 ± 0.65*	0.21 ± 0.06*	17.39± 0.71*
		1 mg/ml	18.70 ± 1.84*	0.27 ± 0.04*	18.97± 1.84*
		2 mg/ml	33.02 ± 2.79*	0.18 ± 0.04*	33.20± 3.83*
	48	0.5 mg/ml	7.42 ± 2.14	0.19 ± 0.03*	7.61±2.17
		1 mg/ml	20.49 ± 2.64*	0.22 ± 0.02*	20.71±2.66*
		2 mg/ml	11.52 ± 3.41	0.25 ± 0.02*	11.77±3.43
	72	0.5 mg/ml	18.43 ± 2.09*	0.14 ± 0.03*	18.57±2.12*
		1 mg/ml	21.66 ± 2.64*	0.17 ± 0.03*	21.83±2.67*
		2 mg/ml	9.55 ± 1.79	0.18 ± 0.03*	9.73±1.82
MJ	24	50 μM	19.22 ± 1.21*	0.17 ± 0.02*	19.39± 1.23*
		100 μM	21.74 ± 1.96*	0.10 ± 0.01*	21.84± 1.97*
		200 μM	20.09 ± 1.07*	0.22 ± 0.05*	20.31± 1.12*
	48	50 μM	13.30 ± 1.35*	0.44 ± 0.09	19.39± 1.44*
		100 μM	23.41 ± 2.66*	0.27 ± 0.07*	13.74± 2.73*
		200 μM	23.50 ± 1.11*	0.46 ± 0.09	23.68± 1.20*
	72	50 μM	18.68 ± 2.31*	0.24 ± 0.03*	23.96± 2.34*
		100 μM	19.70 ± 1.61*	0.21 ± 0.05*	18.92± 1.65*
		200 μM	19.61 ± 2.61*	0.17 ± 0.03*	19.91± 2.64*
CT	24	10 mg/l	10.47 ± 0.56	0.59 ± 0.08*	11.06± 0.64
		20 mg/l	11.75 ± 3.45	0.32 ± 0.04	12.07± 3.49
		50 mg/l	9.60 ± 2.10	0.25 ± 0.04	9.85± 2.14
	48	10 mg/l	9.75 ± 1.97	0.23 ± 0.02*	9.98± 1.89
		20 mg/l	8.41 ± 0.46	0.29 ± 0.07	8.70± 0.53
		50 mg/l	7.82 ± 0.56*	0.21 ± 0.05*	8.03±0.6 0*
		10 mg/l	5.96 ± 2.19*	0.22 ± 0.02*	6.18± 2.21*

Table 8 Effects of elicitors on MuA accumulations in cell cultures of *M. alba* (cont., n=3,* indicates significantly different from control group at the $P<0.01$)

Elicitor	Exposure time (h)	Dose	MuA accumulation (mg/g DW)		
			Intracellular	Extracellular	Total
CT	72	20 mg/l	8.10 ± 2.30	0.20 ± 0.06*	8.30± 2.36
		50 mg/l	7.72 ± 3.61	0.29 ± 0.02	8.00± 3.63
SA	24	50 µM	23.11 ± 2.38*	0.28 ± 0.05	23.39± 2.43*
		100 µM	36.98 ± 1.49*	0.97 ± 0.05*	37.95± 1.54*
		200 µM	33.04 ± 4.37*	1.00 ± 0.16*	34.04± 4.53*
	48	50 µM	19.56 ± 2.29*	0.25 ± 0.04	19.81± 2.33*
		100 µM	21.72 ± 0.82*	0.34 ± 0.06	22.06± 0.88*
		200 µM	12.11 ± 2.23	0.34 ± 0.06	12.45± 2.39
	72	50 µM	16.83 ± 1.19*	0.14 ± 0.02*	16.97± 1.21*
		100 µM	25.53 ± 2.89*	0.19 ± 0.03*	25.72± 2.92*
		200 µM	16.96 ± 1.81*	0.16 ± 0.02*	17.12± 1.83*
Control	-	-	9.91 ± 1.01	0.33 ± 0.06	10.24± 1.07
TE	24	1% v/v	49.44 ± 3.76*	0.27 ± 0.05*	50.19± 3.84*
		2% v/v	49.36 ± 4.32*	0.93 ± 0.26*	50.52± 4.52*
		3% v/v	49.96 ± 5.50*	1.16 ± 0.15*	50.87± 5.65*
	48	1% v/v	37.34 ± 3.61	0.99 ± 0.08*	36.25± 2.16
		2% v/v	38.71 ± 2.16	1.25 ± 0.36*	36.17± 3.61
		3% v/v	35.08 ± 2.98	0.42 ± 0.09*	36.54± 1.82
	72	1% v/v	35.27 ± 2.08	0.74 ± 0.08*	37.61± 3.66
		2% v/v	34.92 ± 3.25	1.16 ± 0.20*	39.64± 2.42
		3% v/v	36.12 ± 1.73	0.91 ± 0.16*	36.24± 3.14
Control	-	-	37.16 ± 2.83	0.13 ± 0.01	37.29± 2.84
BE	24	1% v/v	4.60 ± 0.81	0.74 ± 0.12	5.34± 0.93
		2% v/v	4.20 ± 0.32	1.23 ± 0.03*	5.43± 0.35
		3% v/v	6.50 ± 1.45	2.08 ± 0.12*	8.58± 1.56*
	48	1% v/v	6.00 ± 0.49	0.67 ± 0.09*	6.67± 0.58*

Table 8 Effects of elicitors on MuA accumulations in cell cultures of *M. alba* (cont., n=3,* indicates significantly different from control group at the $P<0.01$)

Elicitor	Exposure time (h)	Dose	MuA accumulation (mg/g DW)		
			Intracellular	Extracellular	Total
BE	48	2% v/v	5.20 ± 0.68	0.78 ± 0.12	5.98± 0.80
		3% v/v	4.83 ± 0.64	0.39 ± 0.03*	5.22± 0.67
	72	1% v/v	8.06 ± 0.54*	2.35 ± 0.33*	10.41± 0.87*
		2% v/v	8.62 ± 0.57*	1.31 ± 0.25	9.93± 0.82*
		3% v/v	6.25 ± 0.64*	0.21 ± 0.06*	6.46± 0.70
Control	-	-	3.87 ± 0.83	0.93 ± 0.13	4.81± 0.96
PE	24	1% v/v	7.16 ± 0.92	0.46 ± 0.11	7.62± 1.03
		2% v/v	8.19 ± 1.16	0.57 ± 0.03	8.76± 1.19
		3% v/v	8.42 ± 1.59	1.42 ± 0.12*	9.84± 1.71*
	48	1% v/v	5.41 ± 0.73	0.74 ± 0.15*	6.15± 0.88
		2% v/v	6.52 ± 0.74	0.21 ± 0.03*	6.73± 0.77
		3% v/v	6.31± 0.70	1.82 ± 0.07*	8.13± 0.77
	72	1% v/v	7.45 ± 0.52	0.49 ± 0.06	7.94± 0.58
		2% v/v	7.24 ± 1.64	0.11± 0.02*	7.35± 1.66
		3% v/v	7.77 ± 1.35	0.66 ± 0.08*	8.43± 1.43
Control	-	-	6.38 ± 1.04	0.50 ± 0.06	6.88± 1.10

2.4 Effects of elicitors on root cultures of *M. alba*

According to growth rate of the root cultures are slower than cell suspension, only selected concentrations of well-known elicitors and microbial extracts were added on 20 days old root cultures (selected concentrations and exposure time are base on previous effects of elicitors on cell cultures results). Figure 22 shows that addition of YE at 2 mg/ml with 24 h exposure time exhibited the highest elicitation effect on total MuA production in root cultures (6.56 folds). The total MuA levels were also significantly increased by addition of MJ, SA,TE, PE and BE (1.70, 1.20,

1.27, 1.26 and 1.37 folds, respectively). Extracellular MuA accumulations were significantly increased in almost elicitors treated groups except PE treated group. Among them, addition of YE showed the highest extracellular MuA level (10.29 folds, compared with control, Table 9).

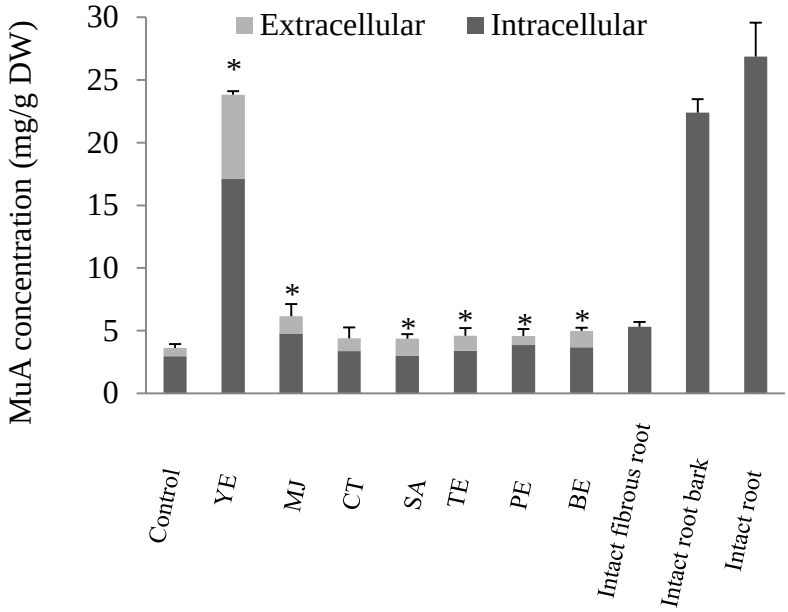

Figure 22 Effects of elicitors on MuA accumulations of root cultures (n=3, * indicates significantly different from control group)

Table 9 Effects of elicitors on MuA accumulations in root cultures of *M. alba*

Elicitor	Exposure time (h)	Dose	MuA accumulation (mg/g DW)		
			Intracellular	Extracellular	Total
YE	24	2 mg/ml	17.12±0.20*	6.69 ± 0.08*	23.81 ±0.28*
MJ	24	200 µM	4.78 ± 0.75*	1.38 ± 0.22*	6.16 ±0.97*
CT	24	10 mg/l	3.38 ± 0.67	1.02 ± 0.20*	4.40 ± 0.87
SA	24	200 µM	3.03 ± 0.25	1.34 ± 0.11*	4.37 ±0.36*
TE	24	1% v/v	3.41 ± 0.46	1.19 ± 0.16*	4.60 ±0.62*
PE	24	3% v/v	3.87 ± 0.46*	0.71 ± 0.08	4.58 ±0.54*
BE	24	3% v/v	3.69 ± 0.18*	1.30 ± 0.06*	4.99 ±0.24*
Control	-	-	2.98 ± 0.26	0.65 ± 0.06	3.63 ± 0.32

Well-known elicitors and aqueous extracts of microbial isolated from *M. alba* were prepared, then added to *in vitro* cultures of *M. alba* in highly growth and high MuA production period.

These results show the addition of YE at 2 mg/ml with 24 h exposure time exhibited the highest elicitation effect on total MuA production in root cultures (23.81±0.30 mg/g DW, increased 6.56 folds) and high elicitation effect on total MuA production in cell suspension cultures (33.02 ± 2.79 mg/g DW, increased 3.24 folds) compared with control. The highest elicitation effect on total MuA production in cell cultures was obtained by addition of 100 µM SA with 48 h exposure time (37.95±1.54 mg/g DW, increased 3.71 folds, compared with control). The total MuA levels in root culture with elicitation by 2 mg/ml YE is higher than that found in intact fibrous root (5.32±0.37 mg/g DW) and similar level as MuA content from root and root bark of several years old intact *M. alba* (22.39±1.06 and 26.86±2.69 mg/g DW, respectively). The concentrations of MuA levels in cell cultures with elicitation by 2 mg/ml YE and 100 µM SA are also higher than MuA levels found in the intact *M. alba* plant (Table 5, 8 and 9). The concentration of intact root bark in this study was founded in the same range with the previous reported, which MuA levels in root bark of mulberry were reported in the range from 0.09 to 53.97 mg/g DW (Piao et al., 2010).

The *in vitro* cultures of *M. alba* with MJ, SA, PE and BE extracts as elicitors significantly enhanced total MuA production, while *Trichoderma* sp. extract significantly increased total MuA productions of cell cultures but not in root cultures. Contrary, CT not significantly increased total MuA production. Mostly, the optimal exposure time of elicitation for both cell and root cultures of *M. alba* is 24 h (Table 8).

The highest extracellular MuA concentration in the cell cultures was obtained by addition of TE (increased 9.61 folds). While, the highest extracellular MuA concentration in root culture was obtained by addition of 2 mg/ml YE (increased 10.29 folds). The percentages of the highest extracellular MuA enhancements are higher than intracellur MuA. However, these results indicate the extracellular MuA concentrations are generally lower than intracellular MuA concentrations.

This study shows that addition of the microbial extracts were also significantly increased MuA levels. These results indicate positive effects from endophytes and

common microbial on secondary metabolites production of their host in the same way with previous report (Wu et al., 2007; Orlita et al., 2008; Wang et al., 2001). Similarly, YE, MJ and SA were reported as elicitors of stilbenes (Belhadj et al., 2006; Roat, Ramawat, 2009).

The significant difference of MuA levels between each experiment were occurred according to some of *in vitro* cultures was subcultured after high MuA production period. Therefore, the MuA accumulations were systematically decreased as show in growth curve of *in vitro* cultures (Figure 12 and 14). In addition, different weights between groups were suggested. Effects of cell density may be affected MuA accumulations in samples. However, each studies of the *in vitro* cultures was obtained from the same batch with no significant differences of final fresh and dry weight within group.

These elicitation studies did not focus on the best condition for MuA production. Optimization of various factor such as using combination of elicitors, inoculums density, nutrient content and light condition possible to increase MuA yield. However, 3 weeks old *in vitro* cultures of *M. alba* with elicitation (24 h exposure time) can produce similar or higher yield of MuA compared with several years old intact explants of *M. alba.*

2.5 Effects of cell density on MuA production of *in vitro* cultures of *M. alba*

To investigate effects of cell density, the different initial fresh weights of cell suspension cultures were transferred to 30 ml medium. Table 10 shows significantly enhancing of MuA levels in high cell density cultures (9 and 12 g initial weight), while 1-6 g initial weights were accumulated 22.35-27.23 mg/g DW MuA. The highest MuA level was obtained from the cell culture with approximately 9 g initial FW (total MuA concentration 82.69±3.76 mg/g DW, total MuA accumulation 73.59±7.25 mg/flask).

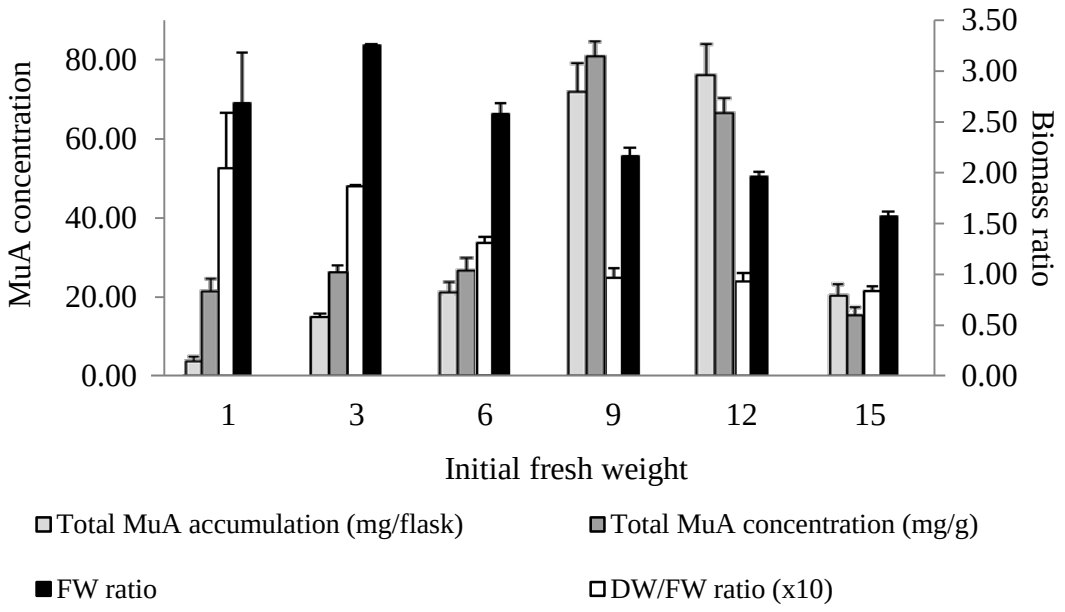

Figure 23 Effects of cell density on total MuA accumulations and biomasses ratio (n=3)

FW and DW/FW ratio were calculated to explain cell density effects on biomass of cell culture. Both FW and DW/FW ratio were significantly decreased when cell density was high (Figure 23). On the contrary, the MuA levels were increased when cell were cultured with high inoculums sizes (Table 10). The 9 g initial FW was considered to be optimal density according to highest MuA accumulation level. Total MuA levels per flask in a cell culture started with 12 g FW is not significant difference with MuA level of the optimal density due to lower MuA yield (66.84±3.98 mg/g DW). On the other hand, the cell culture started with 9 g initial FW can be produced similar MuA level as the cell culture started with 12 g initial FW with lower starter and final biomass. In addition, increasing of cell density also affects extracellular MuA levels in the similar way.

In the same way with previous reported, inoculums sizes of *M. alba in vitro* cultures are also an important factor, that affected secondary metabolites formation and accumulation (Zhang, Zhong, 1997).

Table 10 Effects of cell density on MuA accumulations and biomass (n=3)

Initial fresh weight (g/flask)	MuA concentration (mg/g DW)		FW (g)	DW (g)
	Intracellular	Extracellular		
1	22.35±3.28	0.13±0.04	2.72±0.50	0.20±0.04
3	27.14±1.61	0.03±0.00	9.96±0.04	0.57±0.00
6	27.23±3.19	0.28±0.06	15.59±0.61	0.79±0.03
9	82.26±3.71	0.43±0.05	19.73±0.78	0.89±0.08
12	66.84±3.98	0.64±0.06	23.89±0.61	1.14±0.09
15	16.08±1.91	0.62±0.02	24.13±0.81	1.29±0.06

3. Evaluation of *In Vitro* Cultures and Microorganisms Isolated from *M. alba* for Alpha-Glucosidase Inhibitor Activity and Anti-Tyrosinase Activity

The sterilized surface fibrous roots (Figure 24A) and young twig of *M. alba* (Figure 24B) were plated on LB or PDB solid medium. Various different microorganisms were selected and then subcultured at least 3 times to obtain each single strain.

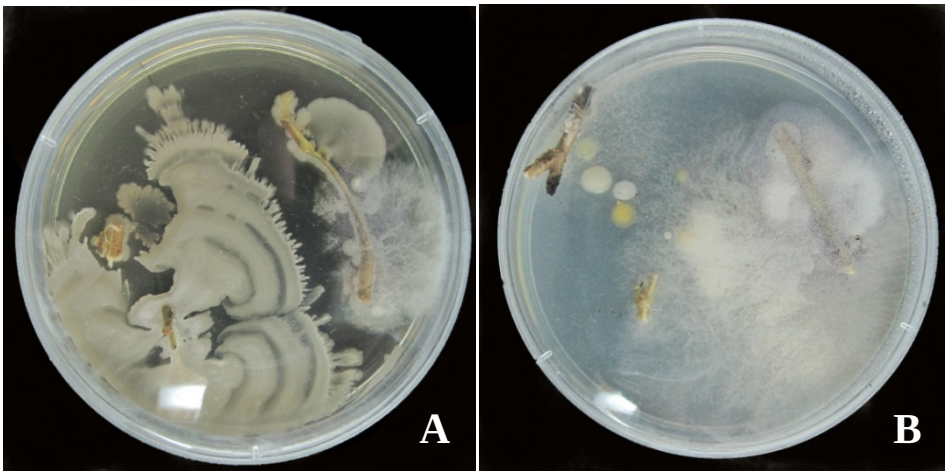

Figure 24 Microbial isolated from *M. alba* on LB (A) and PDB medium (B)

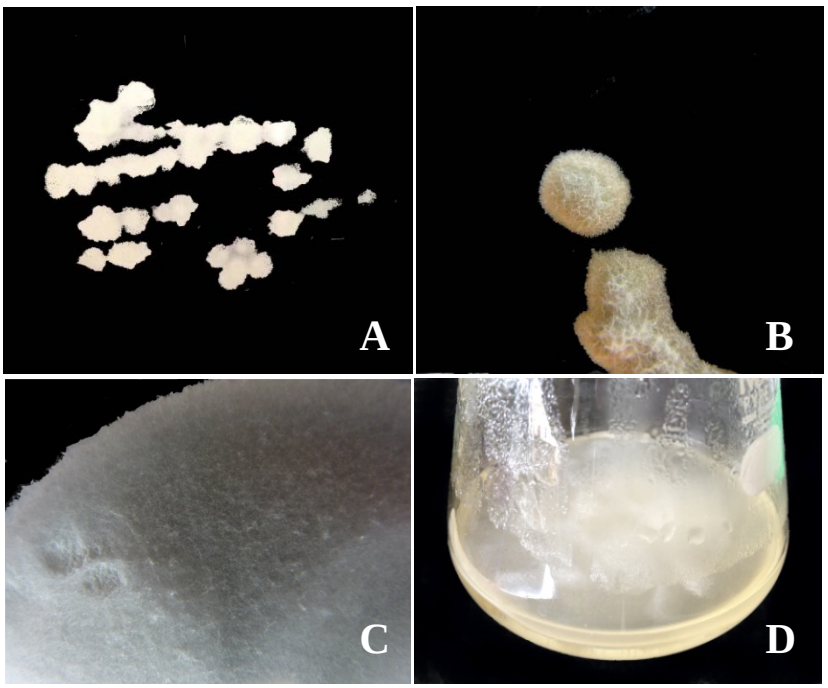

Figure 25 Effective microorganism isolated from *M. alba*, *B. subtillis* (A), *Phoma* sp. (B), and *Trichoderma* sp. on solid (C) and liquid potato dextrose broth (D)

After different microbial were isolated from *M. alba* explants. The microbes were screened for elicitation effects on MuA production from *in vitro* cultures, inhibitory effects on α-glucosidase, tyrosinase activities and MuA accumulation. Three effective elicitation strains were identified as *B. subtillis* (Figure 245), *Phoma* sp. (Figure 25B) and *Trichoderma* sp. (Figure 25C and 25D). Table 11 shows medium extract of endophytic fungus isolated from *M. alba* (identified as *Trichoderma* sp.) can inhibit α-glucosidase (IC50 = 0.81±0.12 mg crude extract/ml) and tyrosinase enzymes (IC50 = 3.72±0.58 mg crude extract/ml). It also showed a positive MuA accumulation (0.40±0.03 mg/g crude extract). While others microbial medium, potato dextrose medium (negative control) and mycelium extracts of *Trichoderma* sp. exhibited the negative results. This study could not conclud that *Trichoderma* sp. can produce MuA, because of the false positive from the ELISA method can be occurred by the cross reactivity of polyclonal antibody. The endophytic fungus may produce compounds with the similar structures as MuA. However, these results still indicated that *Trichoderma* sp. is the interesting source of bioactive secondary metabolites because some microorganisms can produce secondary metabolites related to plants

and level of the secondary metabolites can be improved by optimization of culture conditions. For example, *Bacillus* sp. can produce a 1-deoxynojirimycin and yield of the compound was increased by sorbitol supplementation (Onose et al., 2013). Isolation, identification and optimization of secondary metabolites in the endophytes are interesting points for future study.

Table 11 Inhibitory effects on α-glucosidase and tyrosinase activities and MuA accumulation of the samples (n=3)

Sample	IC_{50} of anti-α-glucosidase activity (mg DW/ml)	IC_{50} of anti-tyrosinase activity (mg DW/ml)	MuA accumulation
Intact fibrous root	0.09±0.01	0.32±0.01	12.40±1.40
Intact root	0.34±0.02	3.20±0.19	14.60±0.85
Intact root bark	0.07±0.01	0.90±0.09	21.05±0.38
Intact twig	0.76±0.07	-	ND
Intact leaf	2.61±0.11	-	ND
Cell culture	0.11±0.01	0.70±0.05	10.31±0.76
Root culture	$86x10^{-6}$±$0.9x10^{-6}$	0.76±0.06	19.34±0.53
Trichoderma sp. medium (ethyl acetate part)	0.81 ± 0.10*	3.72±0.20*	0.40±0.03*
Trichoderma sp. medium (water part)	72.09 ± 3.25*	-	-
1-Deoxynojirimycin	$5.23x10^{-3}$±$0.43x10^{-3}$	-	-
Oxyresveratrol	-	$2.68x10^{-3}$ ±$0.35x10^{-3}$	-

ND = not detected

* = reported in mg crude extract/ml

Table 11 shows the cell suspension and root cultures exhibited a higher level of α-glucosidase inhibition (IC_{50} = 0.11±0.01 mg DW/ml, 86±0.9 ng DW/ml and 0.34±0.02 mg DW/ml, respectively) and tyrosinase inhibition compared with intact root (IC_{50} = 0.70±0.05, 0.76±0.06 and 3.20±0.19 mg DW/ml, respectively). *In vitro* cultures of *M. alba* especially root culture dry powders exerted a strong anti α-glucosidase activity compared with 1-deoxynojirimycin (5.23±0.43 µg DW/ml), one of the most effective iminosugars isolated from *M. alba* (Hunyadi et al., 2012). According to MuA did not show inhibitory activity on the α-glucosidase inhibition assay, for this reason iminosugars, polysaccharide, flavonoids that found in *M. alba* and/or other compounds were suggested to be bioactive compound (s) from *in vitro* cultures. The results indicate that *in vitro* cultures of *M. alba* are interesting sources of MuA, anti-tyrosinase and anti α-glucosidase compounds. Therefore, isolation, identification and optimization of secondary metabolites in the *in vitro* cultures of *M. alba* are also interesting points for the next studies.