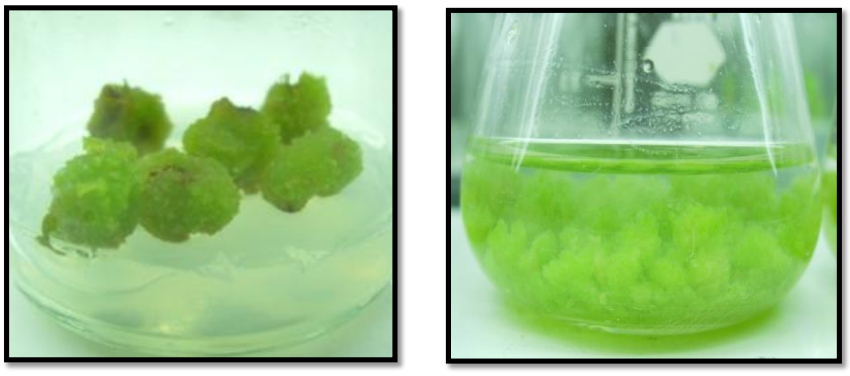
CHAPTER IV
RESULTS

1. Callus and cell suspension culture

1.1 Callus induction

According to the previous study by Udomsuk et al. **(**2011**)** the highest level of total isoflavonoid and chromene contents were achieved from stem explants callus of *P. candollei* var. *mirifica* on MS medium supplemented with 0.1 mg/l TDZ, 1 mg/l NAA and 0.5 mg/l BA. In this experiment, we induced callus from stem of *P. candollei* var. *mirifica* by using MS medium supplemented with 0.1 mg/l TDZ, 1 mg/l NAA and 0.5 mg/l BA as previous study. After subcultured callus of *P. candollei* var. *mirifica* from solid into MS liquid medium with the same combination of plant hormones. The cell suspension was green and friable as show in figure 5. During incubation, the green characteristic of cell suspension gradually turned into brown and some brown substance was secreted from cell into medium after 3–4 weeks of culture.



A **B**

Figure 5 Characteristic of *P. candollei* var. *mirifica* cell suspension culture (A) Callus in solid MS with 0.1 mg/l TDZ, 1 mg/l NAA and 0.5 mg/l BA hormones supplement and (B) Cell suspension in liquid MS medium with 0.1 mg/l TDZ, 1 mg/l NAA and 0.5 mg/l BA hormones supplement

1.2 Growth rate, chromene and total isoflavonoid production in *P. candollei* var. *mirifica* cell suspension culture

After subcultured callus of *P. candollei* var. *mirifica* in to liquid MS medium with 0.1 mg/l TDZ, 1 mg/l NAA and 0.5 mg/l BA. The medium was agitated on a rotary shaker (100 rpm at 25 °C, under continuous light for 16 h/d). Growth rate, chromene and total isoflavonoid production were investigated every 7 days for 28 days. All data were shown in table 2. Cell suspension were harvested and growth rate was determined base on fresh weight and dry weight as see in figure 6. Cell suspension gradually grow during the first week to reach the highest biomass at the last forth week (12.78±2.07 fresh wt g/flask and 0.57±0.05 dry wt g/flask). When considered the secondary metabolite accumulation, chromene and total isoflavonoid production in cell suspension culture were analyzed by HPLC and competitive ELISA, respectively. From figure 7 cell suspension generally produced both chromene and total isoflavonoid from the first week until the last forth week. The highest chromene and total isoflavonoid contents were 45.06±3.50 µg/g dry wt and 2.92±0.64 mg/g dry wt, respectively. These results indicate that during the early third week to the fourth week of stationary phase is the appropriate duration of cell suspension culturing, with ability to produce high active compounds and biomass. The 18 days of cell suspension culture were chosen to treat with elicitors due to the green characteristic of cell suspension gradually turned into brown after 21-day-old.

Table 2 Growth rate, chromene and total isoflavonoid production in *P. candollei* var. *mirifica* cell suspension culture

Day	Fresh wt (g/flask)	Dry wt (g/flask)	Chromene content (µg/g dry wt)	Total isoflavonoid content (mg/g dry wt)
7	6.97 ±1.02	0.29±0.08	23.85±5.34	0.92±0.09
14	6.12 ±0.86	0.36±0.03	34.29±0.58	1.05±0.12
21	9.41 ±2.42	0.57±0.10	40.68±2.54	1.51±0.35
28	12.78 ±2.07	0.57±0.05	45.06±3.50	2.92±0.64

Values represent the mean ± S.D. (n = 3)

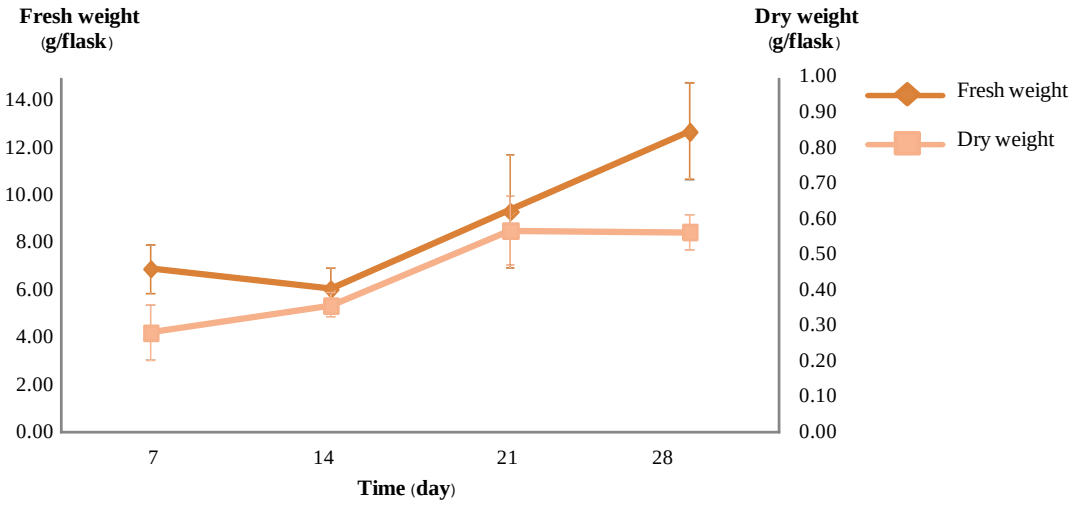


Figure 6 Time course for growth of *P. candollei* var. *mirifica* cell suspension culture in liquid MS medium with 0.1 mg/l TDZ, 1 mg/l NAA and 0.5 mg/l BA hormones supplement for 28 days

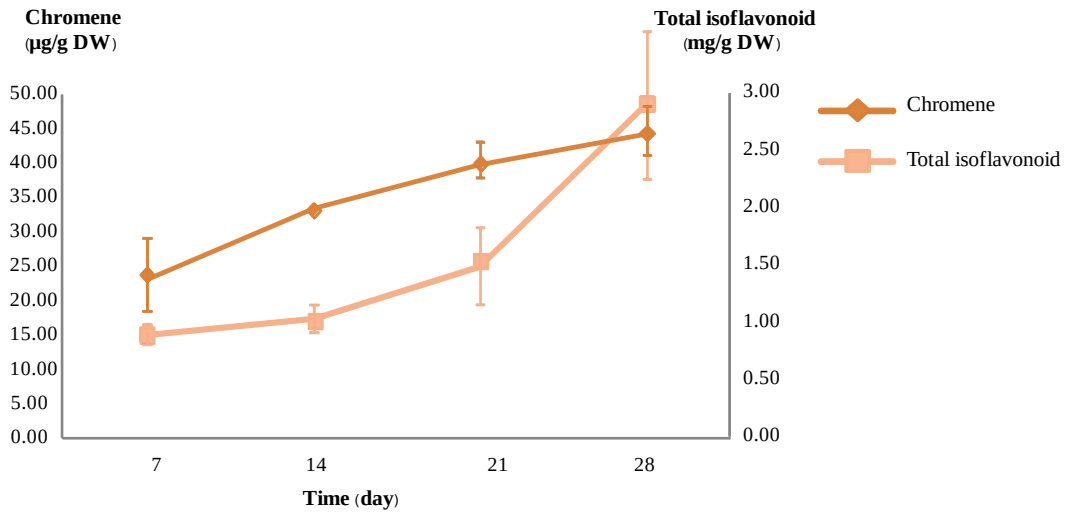


Figure 7 Chromene and total isoflavonoid production in *P. candollei* var. *mirifica* cell suspension culture in liquid MS medium with 0.1 mg/l TDZ, 1 mg/l NAA and 0.5 mg/l BA hormones supplement for 28 days

1.3 Effect of elicitors on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* cell suspension culture

The 18 days of cell suspension culture were treated with various types and concentrations of elicitors. Each elicitor was added in the following final concentration: methyl jasmonate 50, 100 and 200 µM; yeast extract 0.5, 1.0 and 2.0 mg/ml; chitosan 0.5, 1 and 10 mg/l. The medium was agitated on a rotary shaker (100 rpm at 25 °C, under continuous light for 16 h/d). Then the cell suspension were harvested at 3 and 6 days after exposing with elicitors. All treatments were performed in triplicate**.** Chromene and total isoflavonoid contents were determined by HPLC and competitive ELISA, respectively. There was no significant difference in biomass production between the control and all the treatment groups indicate that elicitors were not effect to the growth of cell suspension during elicitation.

1.3.1 Effect of methyl jasmonate on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* cell suspension culture

Cell suspension culture were added 50, 100 and 200 µM methyl jasmonate and harvested at 3 and 6 days after exposing with elicitors. Chromene and total isoflavonoid contents were determined as shown in table 3. The results in figure 8A show that only 200 µM methyl jasmonate significantly increased chromene production after 3 days of elicitation (11.61±0.55 µg/g dry wt, 2-fold higher than control). Figure 8B indicates that 50 µM methyl jasmonate significantly increased total isoflavonoid production after 3 days of elicitation (4.40±0.29 mg/g dry wt, 2-fold higher than control). There was no significant difference on chromene and total isoflavonoid production between other concentrations of methyl jasmonate-treated groups and control group at day 6.

Table 3 Effect of methyl jasmonate on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* cell suspension culture

Elicitor	**Concentration of elicitor**	**Chromene content (µg/g dry wt)**		**Total isoflavonoid content (mg/g dry wt)**	
		Duration (day)		**Duration (day)**	
		3	**6**	**3**	**6**
Control		6.40±0.76	6.86±0.15	2.92±0.41	2.46±0.30
Methyl jasmonate	50 µM	7.02±1.25	5.09±0.43	4.40±0.29	2.51±0.22
	100 µM	5.76±0.60	6.43±0.37	2.73±0.47	3.21±0.99
	200 µM	11.61±0.55	4.94±1.12	3.05±0.48	1.85±0.14

Values represent the mean ± S.D. (n = 3)

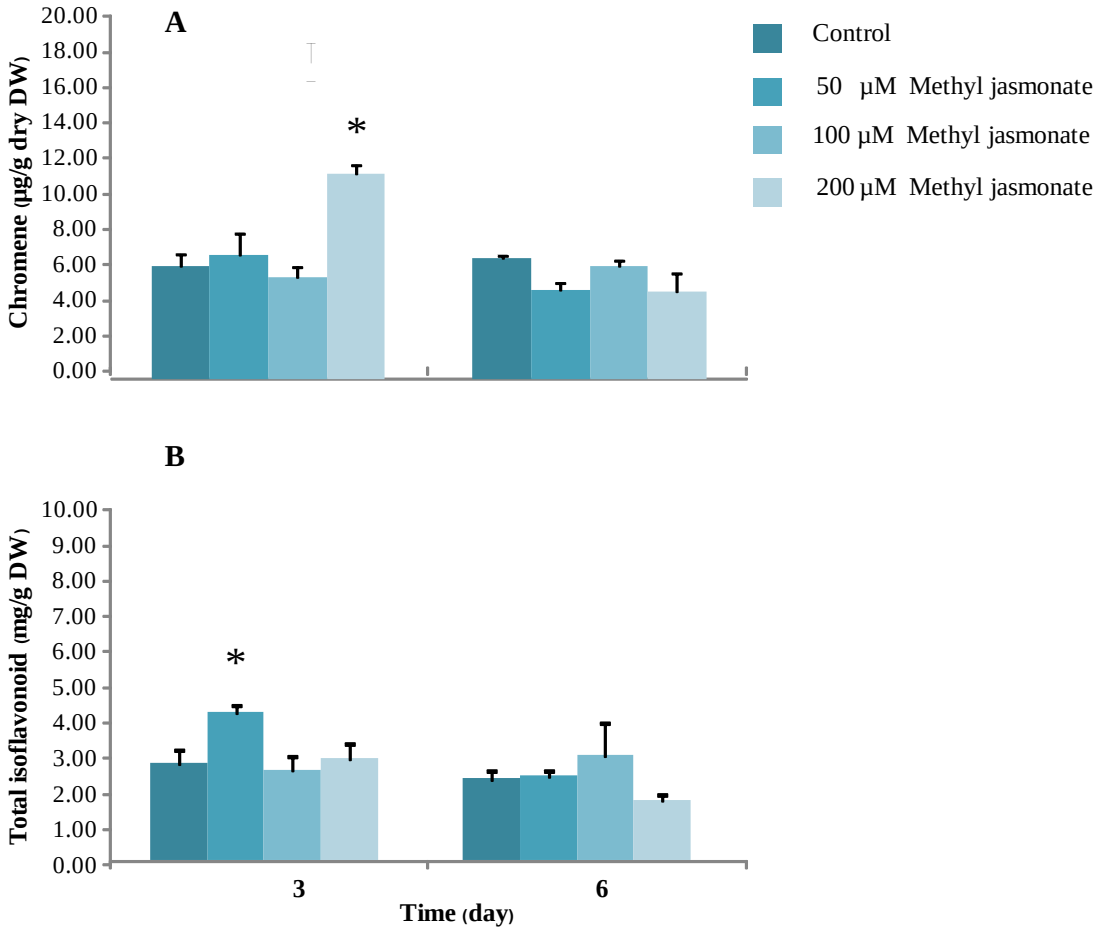


Figure 8 Effect of methyl jasmonate on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* cell suspension culture after 3 and 6 days of elicitation ($p \leq 0.01$)

1.3.2 Effect of yeast extract on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* cell suspension culture

Cell suspension culture were added 0.5, 1 and 2 mg/ml yeast extract and harvested at 3 and 6 days after exposing with elicitors. Chromene and total isoflavonoid contents were determined as shown in table 4**.** Our results in figure 9A indicate that 0.5 and 1 mg/ml yeast extract significantly increased chromene production after 3 days of elicitation. Adding of 1 mg/ml yeast extract could elicit the highest of chromene production after 3 days of elicitation (7.84±0.53µg/g dry wt,

1.4-fold higher than control). There was no significant difference on chromene production between yeast extract-treated groups and control group at day 6.

Figure 9B showed that almost treatments with various concentrations of yeast extract significantly increased the production of total isoflavonoid after 3 days and 6 days of elicitation. Especially addition of 0.5 mg/ml yeast extract for 6 days showed the highest enhancing of total isoflavonoid content in cell suspension culture (9.61±0.50 mg/g dry wt, 3-fold higher than control).

Table 4 Effect of yeast extract on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* cell suspension culture

Elicitor	**Concentration of elicitor**	**Chromene content (µg/g dry wt)**		**Total isoflavonoid content (mg/g dry wt)**	
		Duration (day)		**Duration (day)**	
		3	**6**	**3**	**6**
Control		5.03±0.55	5.04±0.41	2.46±0.44	3.39±0.19
Yeast extract	0.5 mg/ml	6.49±0.47	3.34±0.33	5.26±0.66	9.61±0.50
	1 mg/ml	7.84±0.53	4.77±0.60	4.42±0.24	3.92±0.57
	2 mg/ml	5.25±0.42	3.39±0.24	4.45±0.18	5.78±0.45

Values represent the mean ± S.D. (n = 3)

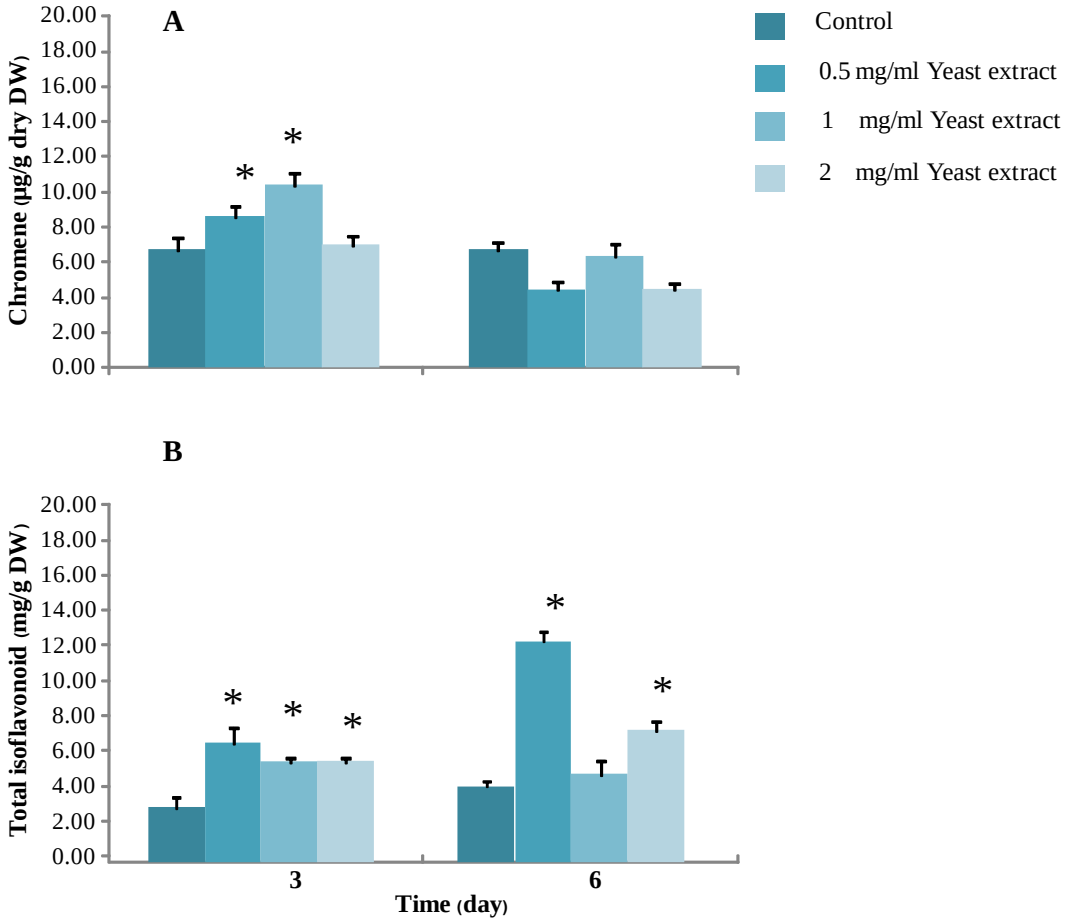


Figure 9 Effect of yeast extract on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* cell suspension culture after 3 and 6 days of elicitation ($p \leq 0.01$)

1.3.3 Effect of chitosan on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* cell suspension culture

Cell suspension culture were added 0.5, 1 and 10 mg/l chitosan and harvested at 3 and 6 days after exposing with elicitors. Chromene and total isoflavonoid contents were determined and also shown in table 5. Figure 10A indicates that 0.5 and 1 mg/l chitosan significantly increased chromene production after 6 days of elicitation (6.58±0.49 and 6.10±0.72 µg/g dry wt, respectively). There was no significant difference on chromene production between chitosan-treated groups and control group at day 3. Figure 10B shows that addition 0.5 and 2 mg/l

chitosan significantly increased total isoflavonoid production after 3 days (3.54±0.28 and 4.16±0.64 mg/g dry wt, respectively) and 6 days of elicitation (7.41±0.66 and 5.54±0.59 mg/g dry wt, respectively). Addition of 0.5 mg/l chitosan for 6 days showed the highest enhancing of total isoflavonoid content in cell suspension culture (7.41±0.66 mg/g dry wt., 2-fold higher than control).

Table 5 Effect of chitosan on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* cell suspension culture

Elicitor	**Concentration of elicitor**	**Chromene content (µg/g dry wt)**		**Total isoflavonoid content (mg/g dry wt)**	
		Duration (day)		**Duration (day)**	
		3	**6**	**3**	**6**
Control		5.03±0.55	5.04±0.41	2.46±0.44	3.39±0.19
Chitosan	0.5 mg/l	3.49±0.35	6.58±0.49	3.54±0.28	7.41±0.66
	1 mg/l	3.76±0.65	6.10±0.72	2.61±0.17	4.08±0.39
	10 mg/l	3.28±0.18	4.60±0.40	4.16±0.64	5.54±0.59

Values represent the mean ± S.D. (n = 3)

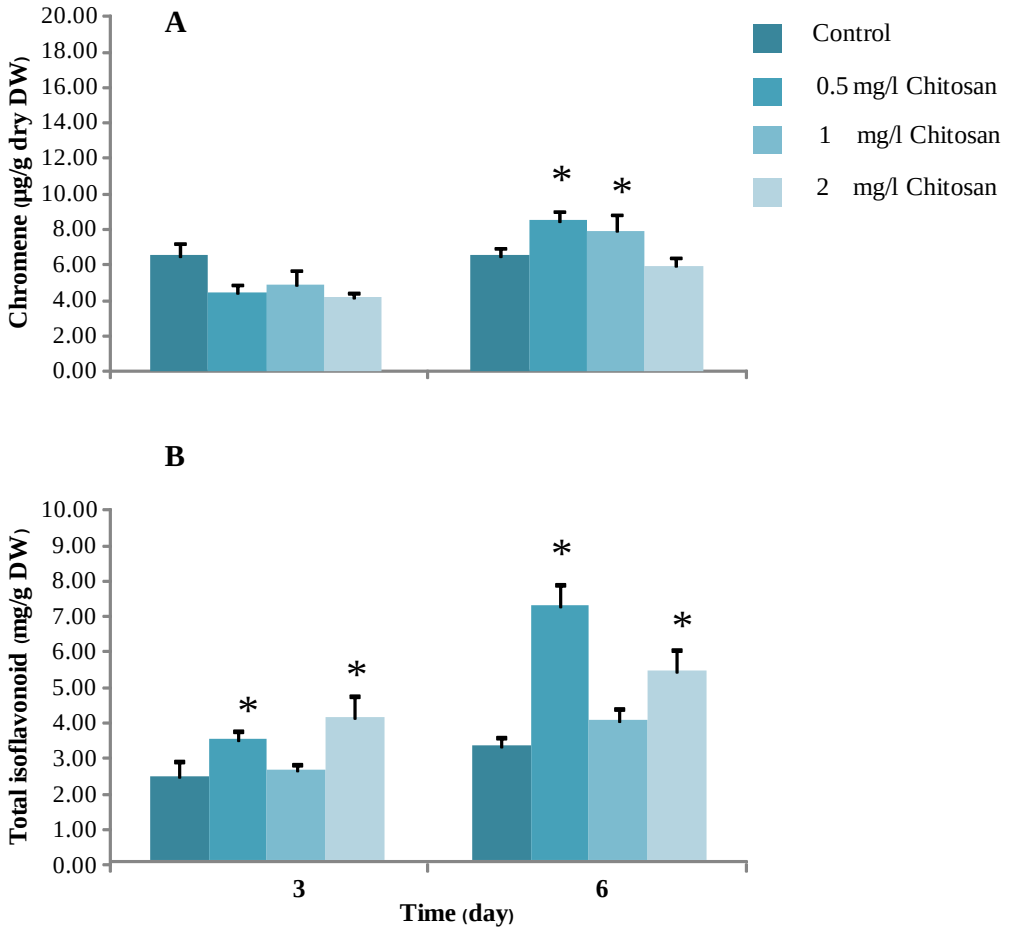


Figure 10 Effect of chitosan on chromene and total isoflavonoid production in *P.candollei* var. *mirifica* cell suspension culture after 3 and 6 days of elicitation ($p \leq 0.01$)

2. Hairy root culture

2.1 Hairy root induction

Cotyledon, leaves and stems part of *P. candollei* var. *mirifica* were infected with *Agrobacterium rhizogenes* ATCC 15834 and transferred to ½MS medium containing 500 mg/l cefotaxime. Hairy roots were emerged from infection sites and observed within 14 days. Two weeks interval, the hairy roots were subculture on ½ MS medium containing 400, 300, 200, 100 mg/l cefotaxime and ½MS medium, respectively. Transformed roots were grown in 125-ml flasks containing 30 ml of

½ MS liquid medium and subculture every 2 weeks into fresh medium. The medium was agitated on a rotary shaker (100 rpm at 25 °C, under continuous light for 16 h/d). Characteristics of transformed root were fast growth and lateral branching as shown in figure 11. During incubation, young hairy roots with white color were gradually turned into brown and some brown substance was secreted from hairy roots into medium after 3-4 weeks of culture.

When compare hairy root of *P. candollei* var. *mirifica* and *P. candollei* var. *candollei* which was established by Udomsuk et al. (2009), hairy root of *P. candollei* var. *mirifica* had some difference characteristic but both were fast growth and lateral branching. *P. candollei* var. *mirifica* had hard fragile texture while *P. candollei* var. *candollei* had soften look like spongy texture as shown in figure 12. These results were similar to previously studied that found in hairy roots culture of *P. candollei* var. *mirifica* (Thanonkeo, 2006).

A **B** **C**

Figure 11 Characteristic of *P. candollei* var. *mirifica* hairy root in (A) solid ½ MS, (B) and (C) liquid ½ MS medium

A **B**

Figure 12 Hairy root of *P. candollei* var. *mirifica* (A) and *P. candollei* var. *candollei* (B)

2.2 Growth rate, chromene and total isoflavonoid production in *P. candollei* var. *mirifica* hairy root culture

After transferred hairy root from solid ½ MS in to liquid ½ MS medium, growth rate, chromene and total isoflavonoid production of hairy root were investigated every 7 days for 35 days as shown in table 6. Growth rate of hairy root was determined by fresh weight and dry weight as see in figure 13. Hairy root gradually grow up during the first week to the forth week and reach to the highest at the last forth week (1.61±0.25 fresh wt g/flask and 0.11±0.02 dry wt g/flask) then the growth rate was decreased. In term of investigate secondary metabolite accumulation, chromene and total isoflavonoid production in hairy root culture were analyzed by HPLC and competitive ELISA, respectively. In figure 14, hairy root generally produced both chromenes and total isoflavonoid from the first week until the last third week which producing the highest chromene content 9.17±0.73 μg/g dry wt and total isoflavonoid content 9.56±0.29 mg/g dry wt. After the last third week production of both active compounds were decreased. These results indicate that 3 weeks of culture is appropriate period for the highest active compounds and biomass production. Thus 21 days of hairy root culture (an exponential or log phase) was chosen for further study as previous report by Udomsuk et al. (2009).

Table 6 Growth rate, chromene and total isoflavonoid production in *P. candollei* var. *mirifica* hairy root culture

Day	Fresh wt (g/flask)	Dry wt (g/flask)	Chromene content (µg/g dry wt)	Total isoflavonoid content (mg/g dry wt)
7	0.54±0.10	0.04±0.01	6.86±0.44	5.97±0.32
14	1.29±0.18	0.09±0.01	6.60±0.46	6.19±0.65
21	1.50±0.19	0.10±0.01	9.17±0.73	9.56±0.29
28	1.61±0.25	0.11±0.02	3.62±0.30	8.62±0.85
35	1.06±0.13	0.08±0.01	3.93±0.24	11.53±0.60

Values represent the mean ± S.D. (n = 3)

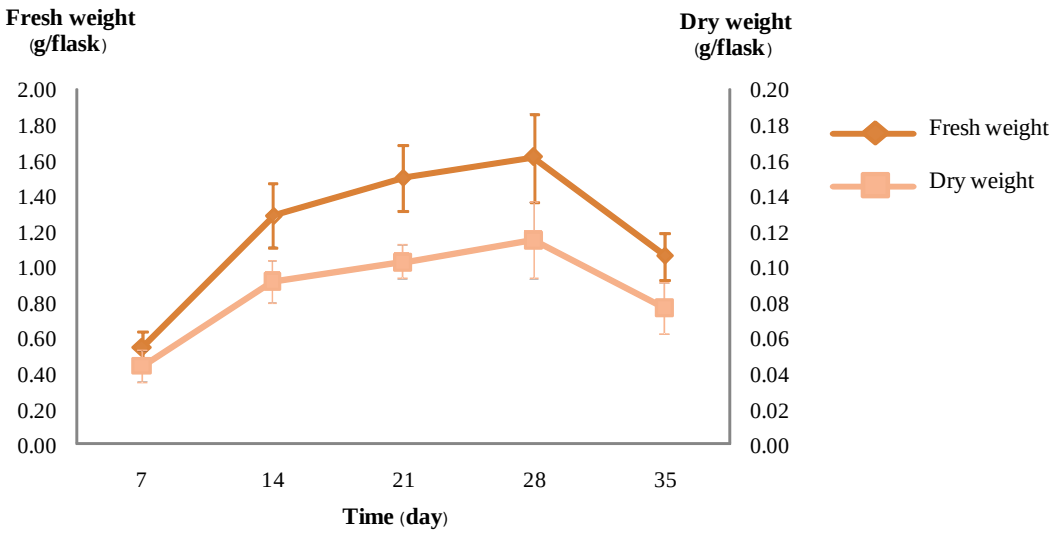


Figure 13 Time course for growth of *P. candollei* var. *mirifica* hairy root culture in ½ MS medium for 35 days

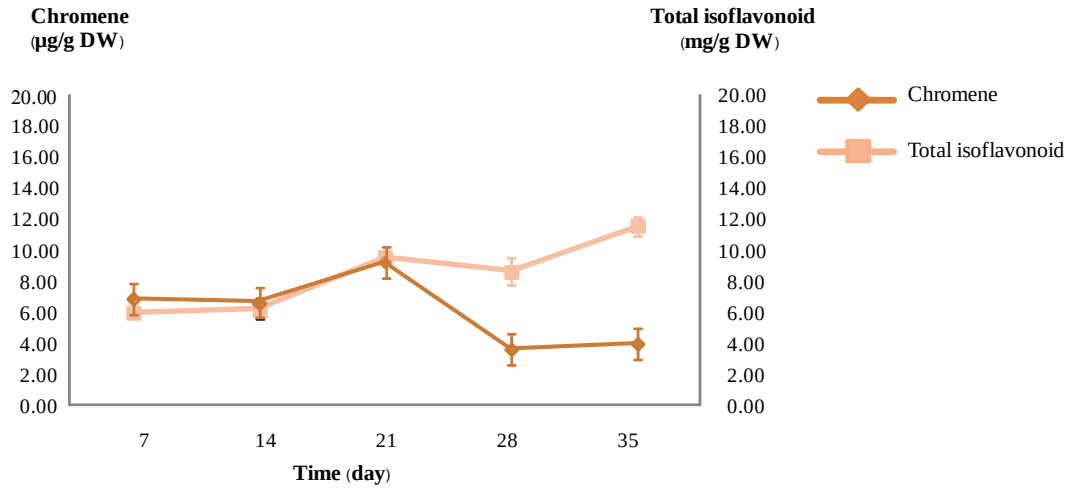


Figure 14 Chromene and isoflavonoid production in *P. candollei* var. *mirifica* hairy root culture in ½ MS medium for 35 days

2.3 Effect of elicitors on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* hairy root

Base on previous data from Udomsuk et al. (2011) studying the effect of elicitors in *P. candollei* var. *candollei* hairy root culture, the best type and concentration of elicitors that elicited the highest total isoflavonoid were chosen. Then 200 µM methyl jasmonate, 0.5 mg/ml yeast extract and 0.5 mg/l chitosan were used as elicitors in this experiment.

21 days transformed hairy roots of *P. candollei* var. *mirifica* in ½ MS liquid medium were added 200 µM methyl jasmonate, 0.5 mg/ml yeast extract or 0.5 mg/l chitosan. The medium was agitated on a rotary shaker (100 rpm at 25 °C, under continuous light for 16 h/d). Then the hairy roots were harvested at 3 and 6 days after exposing with elicitors. All treatments were performed in triplicate**.** There was no significant difference in biomass production between the control and all the treatment groups indicate that elicitors were not effect to the growth of hairy root during elicitation. Chromene and total isoflavonoid production in hairy root culture were analyzed by HPLC and competitive ELISA, respectively. Results were shown in table 7.

Table 7 Effect of elicitors on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* hairy root

Elicitor	Concentration of elicitor	Chromene content (µg/g dry wt)		Total isoflavonoid content (mg/g dry wt)	
		Duration (day)		Duration (day)	
		3	6	3	6
Control (methyl jasmonate and yeast)		10.23±0.58	9.24±1.56	8.48±0.58	7.48±0.251
Methyl jasmonate	200 µM	17.03±1.12	13.31±0.47	16.06±1.28	7.07±0.673
Yeast extract	0.5 mg/ml	12.35±0.70	17.47±0.00	14.65±1.44	9.17±1.164
Control (chitosan)		5.08±0.25	4.35±0.10	4.38±0.25	8.25±0.391
Chitosan	0.5 mg/l	5.54±0.41	4.48±0.15	5.40±0.09	8.44±0.523

Values represent the mean ± S.D. (n = 3)

2.3.1 Effect of methyl jasmonate on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* hairy root culture

Hairy roots of *P. candollei* var. *mirifica* in ½ MS liquid medium were added 200 µM methyl jasmonate and harvested at 3 and 6 days after exposing with elicitors. The results in figure 15A indicate that 200 µM methyl jasmonate significantly increased chromene production after 3 days and 6 days of elicitation (17.03±1.12 and 13.31±0.47 µg/g dry wt, 2 and 1.5-fold higher than control, respectively). On the other hand, figure 15B demonstrates that 200 µM methyl jasmonate highly significantly increased total isoflavonoid production after 3 days of elicitation (16.06±1.28 mg/g dry wt, 2-fold higher than control).

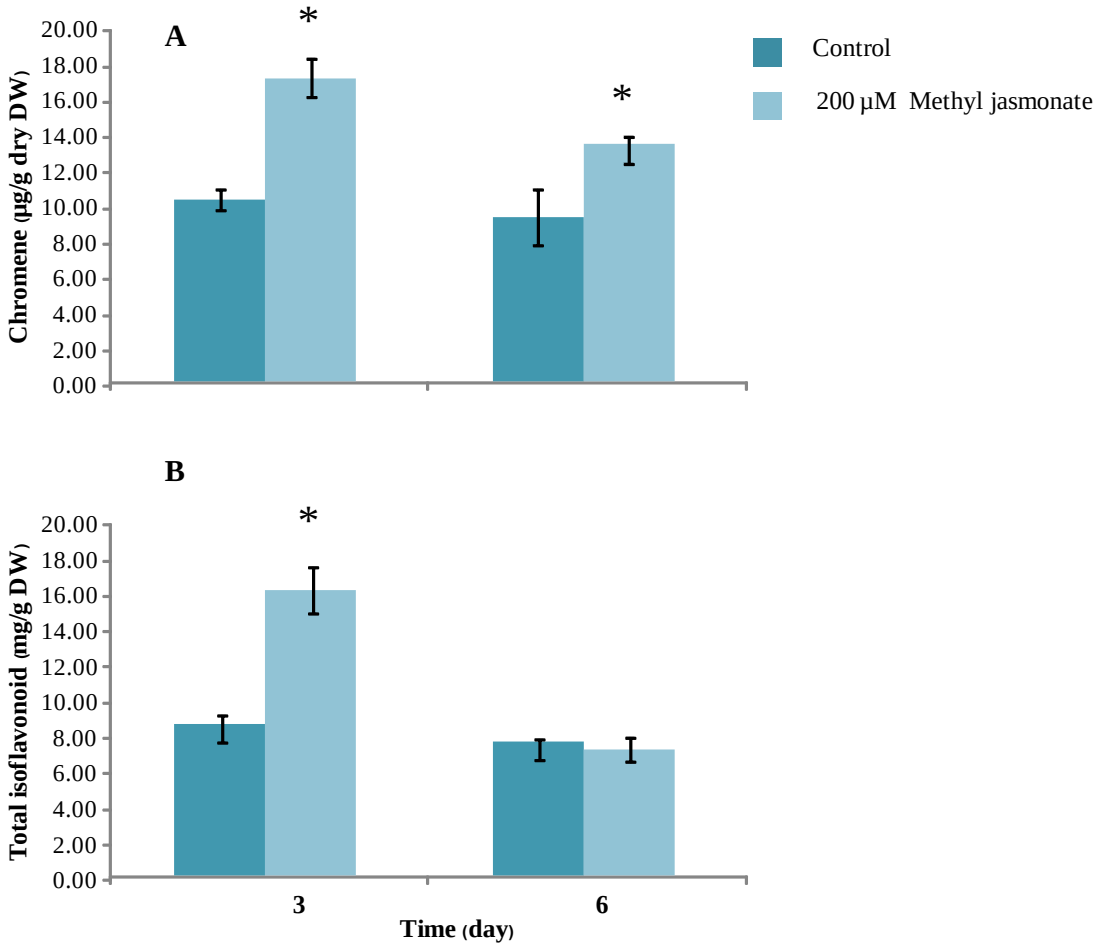


Figure 15 Effect of methyl jasmonate on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* hairy root culture after 3 and 6 days of elicitation ($p \leq 0.01$)

2.3.2 Effect of yeast extract on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* hairy root culture

Hairy roots of *P. candollei* var. *mirifica* in ½ MS liquid medium were added 0.5 mg/ml yeast extract and harvested at 3 and 6 days after exposing with elicitors. The results in figure 16A show that 0.5 mg/ml yeast extract significantly increased chromene production after 3 days and 6 days of elicitation (12.35±0.70 and 17.47±0.00 µg/g dry wt, 2-fold higher than control, respectively). Figure 16B demonstrates that 0.5 mg/ml yeast extract highly significantly increased total

isoflavonoid production after 3 days of elicitation (14.65±1.44 mg/g dry wt, 2-fold higher than control).

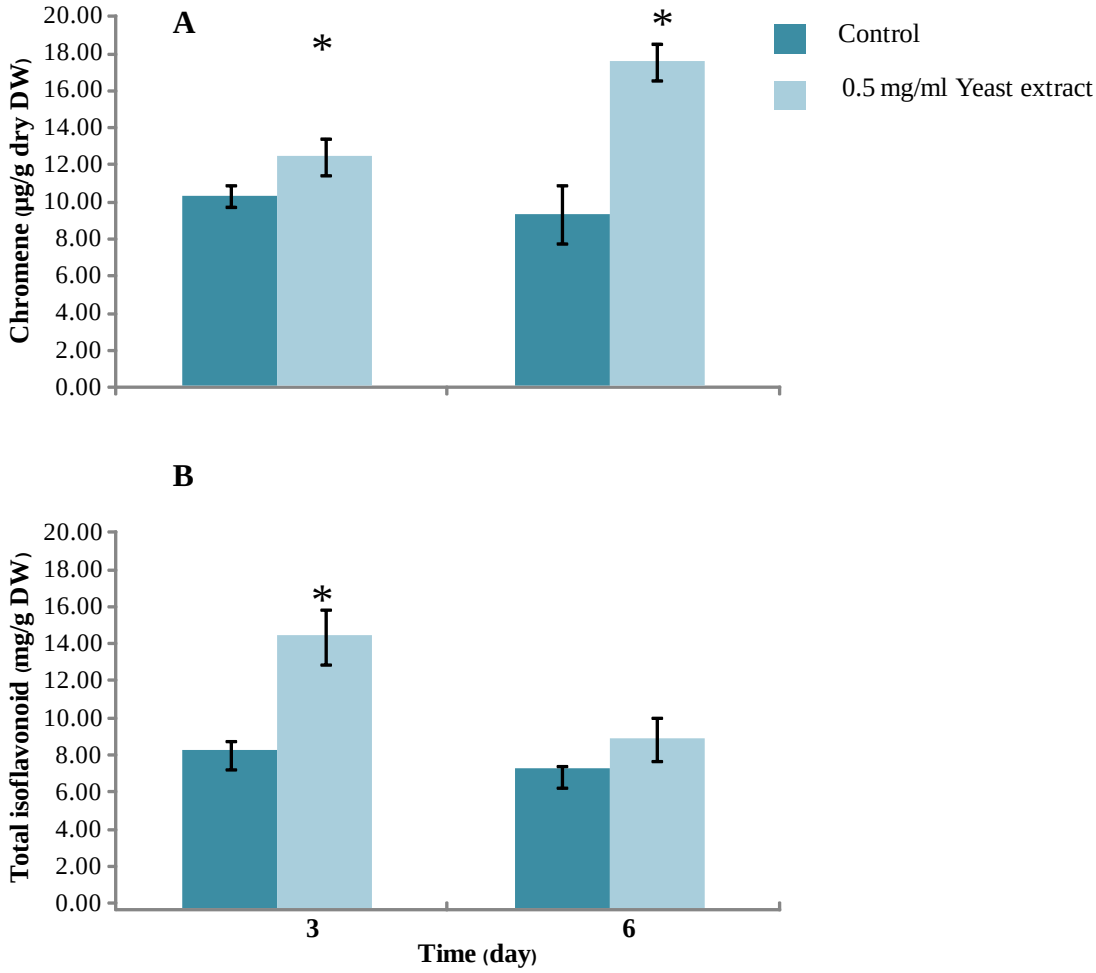


Figure 16 Effect of yeast extract on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* hairy root culture after 3 and 6 days of elicitation ($p \leq 0.01$)

2.3.3 Effect of chitosan on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* hairy root culture

Hairy roots of *P. candollei* var. *mirifica* in ½ MS liquid medium were added 0.5 mg/l chitosan and harvested at 3 and 6 days after exposing with elicitors.

The result in figure 17 did not show any effect of 0.5 mg/l chitosan on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* hairy root culture.

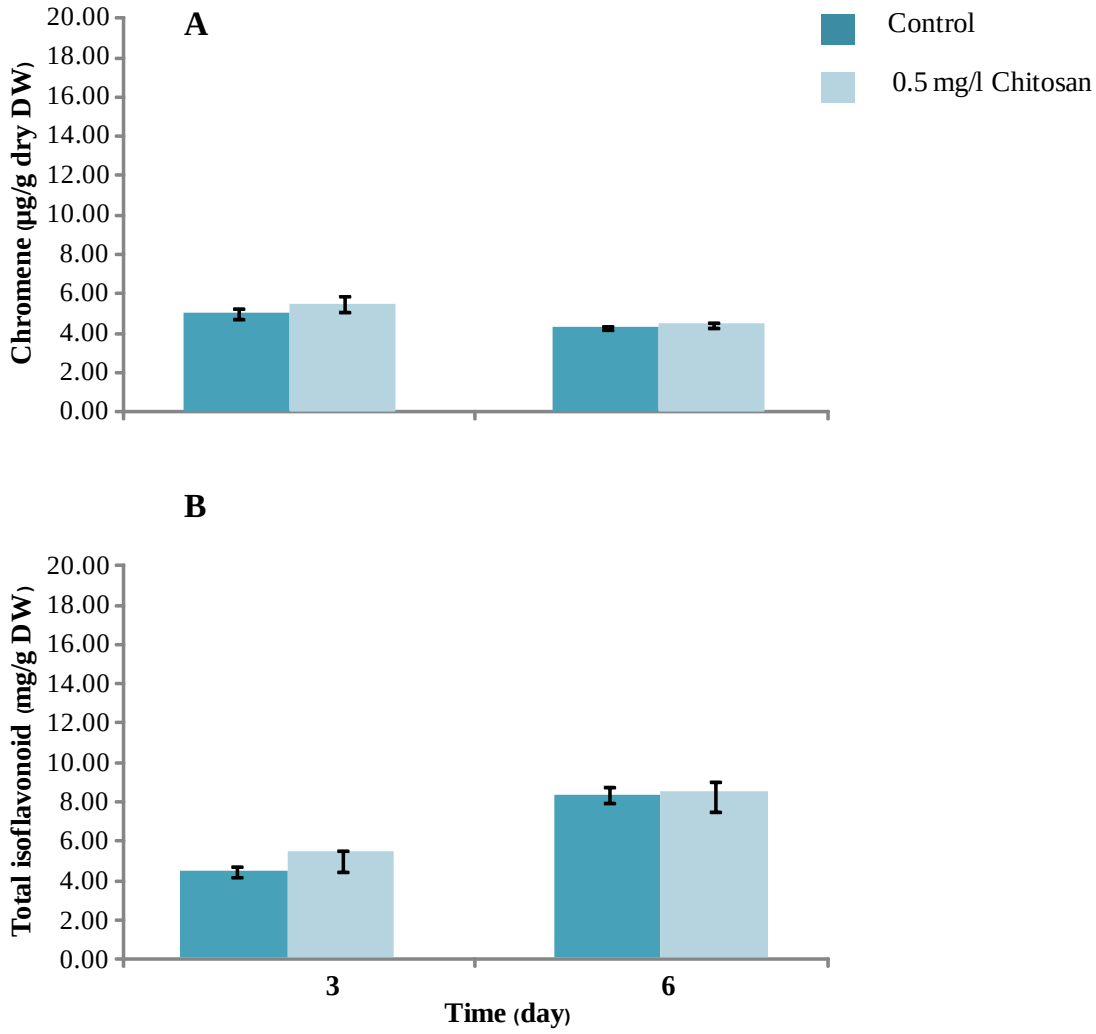


Figure 17 Effect of chitosan on chromene and total isoflavonoid production in *P. candollei* var. *mirifica* hairy root culture after 3 and 6 days of elicitation ($p \leq 0.01$)

2.4 Effect of hormone elicitors on total isoflavonoid production in two varieties of *P. candollei* hairy root culture

In Thailand there are two plants varieties, *P. candollei* var. *mirifica* (PM) and *P. candollei* var. *candollei* (PC) which have similar botanical characteristic but both vary in chemical component containing. We investigated the effects of plant growth regulator N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU), thidiazuron (TDZ) and abscisic acid (ABA) on plant growth and isofavonoid production in two varieties of *P. candollei* hairy root culture.

21 days transformed hairy roots of two varieties *P. candollei* in ½ MS medium were transferred to ½ MS liquid medium which each containing various types and concentrations of plant hormones (CPPU, TDZ at 0.01, 0.05, 0.1 and 0.5 mg/l and ABA at 0.1, 0.5, 1, 2 mg/l). Then the hairy roots were harvested at 5 and 10 days after exposing with hormone. All treatments were performed in triplicate. Total isoflavonoid production in hairy root culture was analyzed by competitive ELISA. There was no significant difference in biomass production between the control and all the treatment groups indicate that hormones were not effect to the growth of hairy root during elicitation.

2.4.1 Effect of N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) on total isoflavonoid production in two varieties of *P. candollei* hairy root culture

Table 8 and figure 18 show that addition various concentrations of CPPU significantly increased the production of total isoflavonoid in PM hairy root culture after 5 days and 10 days of elicitation. Only 0.01 mg/l CPPU at 5 days did not show effect on total isoflavonoid content in PM hairy root cultures. Adding 0.1 mg/l CPPU elicited the highest total isoflavonoid in PM hairy root culture after 5 days of elicitation (22.02±1.30 mg/g dry wt, 4-fold higher than control). CPPU at 0.01 and 0.5 mg/l significantly increased the production of total isoflavonoid in PC hairy root culture after 5 days of elicitation (11.43±0.57 and 11.27±0.34 mg/g dry wt, respectively). However, there was no significant difference between CPPU-treated and control PC roots at day 10.

Table 8 Effect of N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) on total isoflavonoid production in two varieties of *P. candollei* hairy root culture

Elicitor	Concentration of elicitor (mg/l)	Total isoflavonoid content (mg/g dry wt)			
		PM		PC	
		Duration (day)		Duration (day)	
		5	10	5	10
Control		5.47±1.02	7.67±0.13	9.42±0.37	10.75±0.45
CPPU	0.01	5.62±0.90	15.49±0.93	11.43±0.57	7.81±0.57
	0.05	19.73±1.60	13.81±1.41	7.83±0.57	7.76±0.63
	0.1	22.02±1.30	11.42±1.68	7.80±0.53	4.43±0.35
	0.5	12.48±1.18	13.25±0.94	11.27±0.34	4.77±0.88

Values represent the mean ± S.D. (n = 3)

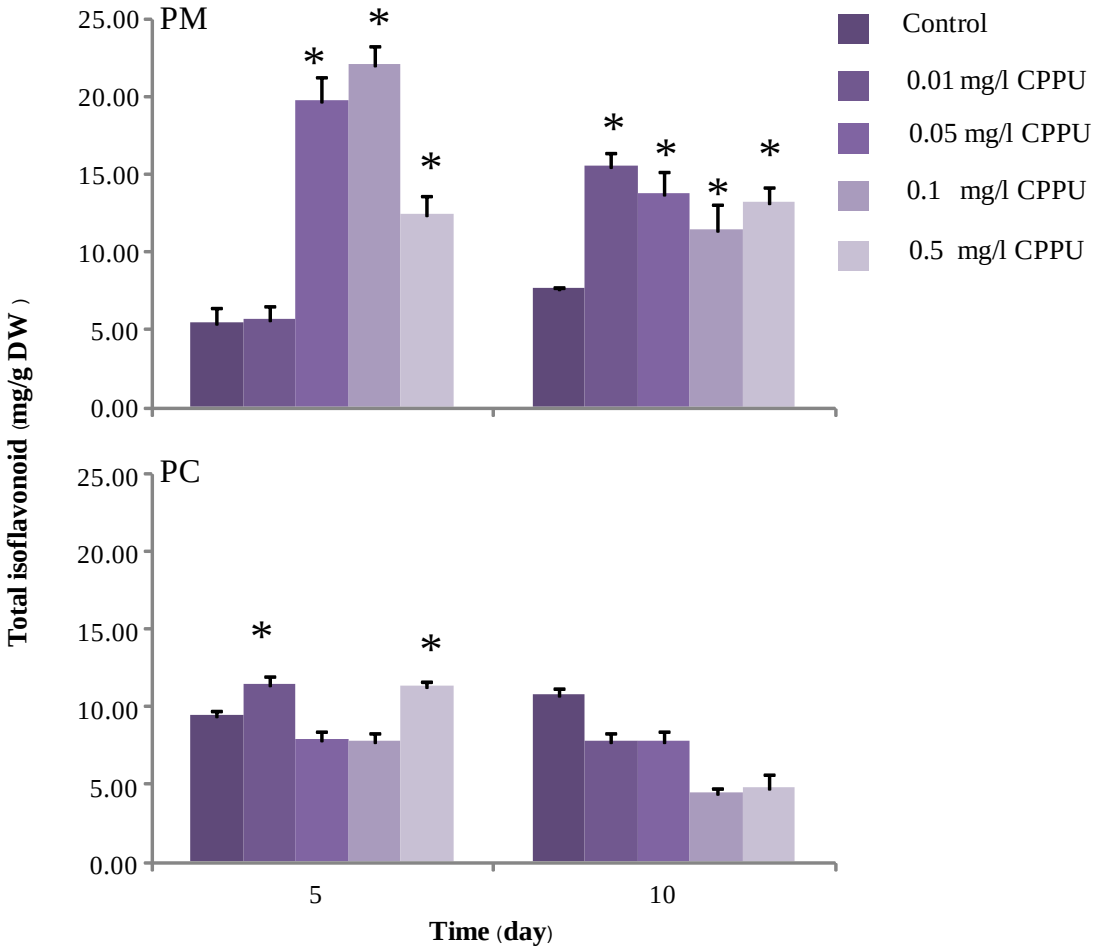


Figure 18 Effect of N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) on total isoflavonoid production in two varieties of *P. candollei* hairy root culture ($p \leq 0.01$)

2.4.2 Effect of thidiazuron (TDZ) on total isoflavonoid production in two varieties of *P. candollei* hairy root culture

Our results show that addition various concentrations of TDZ significantly increased the production of total isoflavonoid in PM hairy root cultures after 5 days of elicitation (table 9 and figure 19). When increasing proportion of TDZ at higher concentration, the total isoflavonoid content decreased after 5 days of elicitation. Adding 0.01 mg/l TDZ could elicit the highest total isoflavonoid production in PM hairy root culture after 5 days of elicitation (22.64±1.00 mg/g dry

wt, 4-fold higher than control). While addition of TDZ 0.05 mg/l significantly increased the highest effect production of total isoflavonoid in PC hairy root after 5 days of elicitation (16.03±0.77 mg/g dry wt, 2-fold higher than control).

Table 9 Effect of thidiazuron (TDZ) on total isoflavonoid production in two varieties of *P. candollei* hairy root culture

Elicitor	**Concentration of elicitor (mg/l)**	**Total isoflavonoid content (mg/g dry wt)**			
		PM		**PC**	
		Duration (day)		**Duration (day)**	
		5	**10**	**5**	**10**
Control		5.47±1.02	7.67±0.13	9.42±0.37	10.75±0.45
TDZ	0.01	22.64±1.00	11.07±0.73	10.91±0.65	13.33±0.19
	0.05	18.16±0.73	10.27±2.66	16.03±0.77	6.59±0.46
	0.1	12.53±0.66	7.13±0.59	9.16±0.58	3.53±0.38
	0.5	11.80±0.61	8.94±2.88	7.18±0.72	4.24±0.56

Values represent the mean ± S.D. (n = 3)

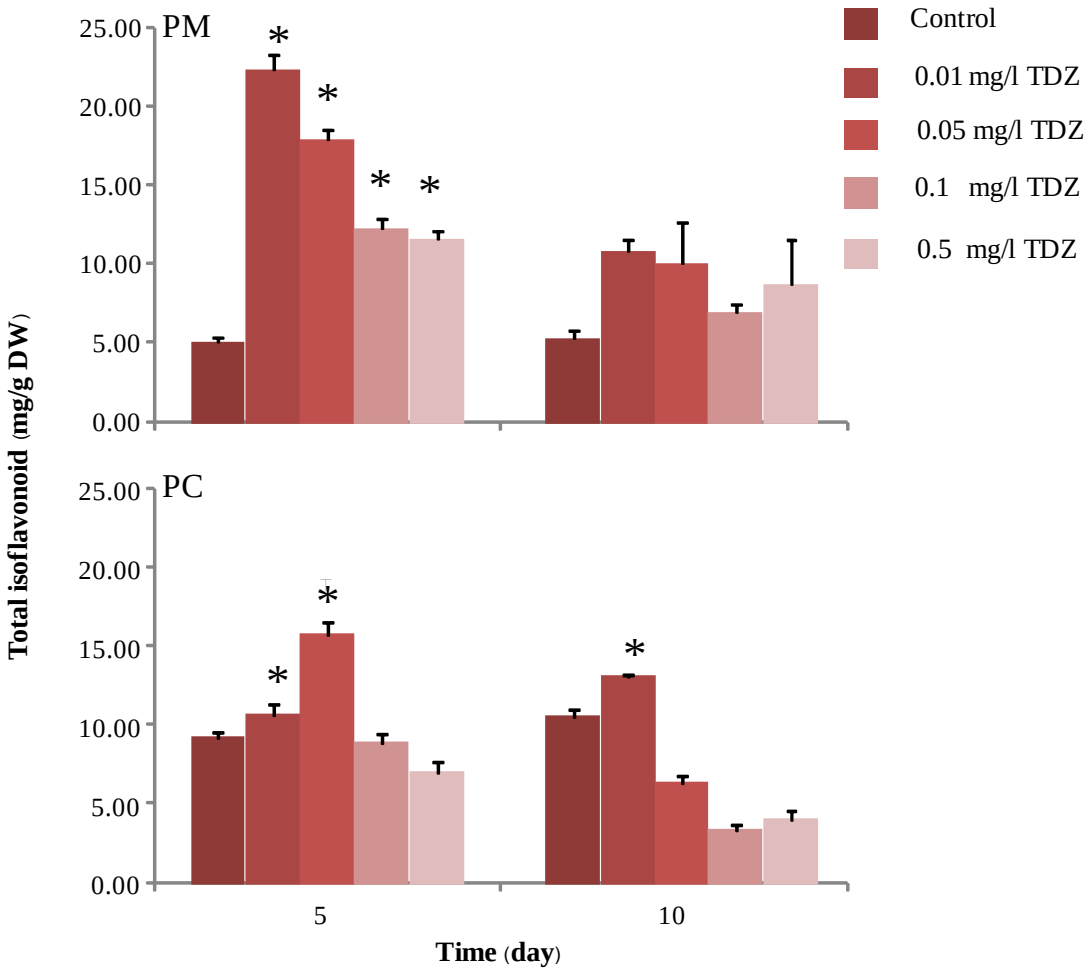


Figure 19 Effect of thidiazuron (TDZ) on total isoflavonoid production in two varieties of *P. candollei* hairy root culture ($p \leq 0.01$)

2.4.3 Effect of abscisic acids (ABA) on total isoflavonoid production in two varieties of *P. candollei* hairy root culture

Table 10 and figure 20 indicate that addition only of ABA 0.1 mg/l for 5 days significantly increased total isoflavonoid content in PM hairy root culture (10.29±0.60 mg/g dry wt, 2-fold higher than control). There was no significant difference between TDZ-treated and control PC roots at day 10. In the other hand, various concentrations of ABA showed significantly increased the production of total isoflavonoid in PC hairy root culture. Addition of ABA 0.1 mg/l for 5 days showed the highest of total isoflavonoid content in PC hairy root (17.90±0.16 mg/g dry wt,

2-fold higher than control). In addition, ABA decreased total isoflavonoid content with increasing proportion of elicitor at higher concentration after 5 days of elicitation.

Table 10 Effect of abscisic acids (ABA) on total isoflavonoid production in two varieties of *P. candollei* hairy root culture

Elicitor	**Concentration of elicitor (mg/l)**	**Total isoflavonoid content (mg/g dry wt)**			
		PM		**PC**	
		Duration (day)		**Duration (day)**	
		5	**10**	**5**	**10**
Control		5.27±0.33	5.42±0.62	9.455±0.841	8.142±0.894
ABA	0.1	10.29±0.60	7.00±0.65	17.90±0.16	10.01±0.21
	0.5	6.80±0.40	6.43±1.20	13.06±0.48	10.72±0.86
	1	6.00±0.67	2.84±0.08	12.19±0.88	10.83±0.85
	2	6.68±0.50	2.32±0.29	10.14±0.65	9.10±0.77

Values represent the mean ± S.D. (n = 3)

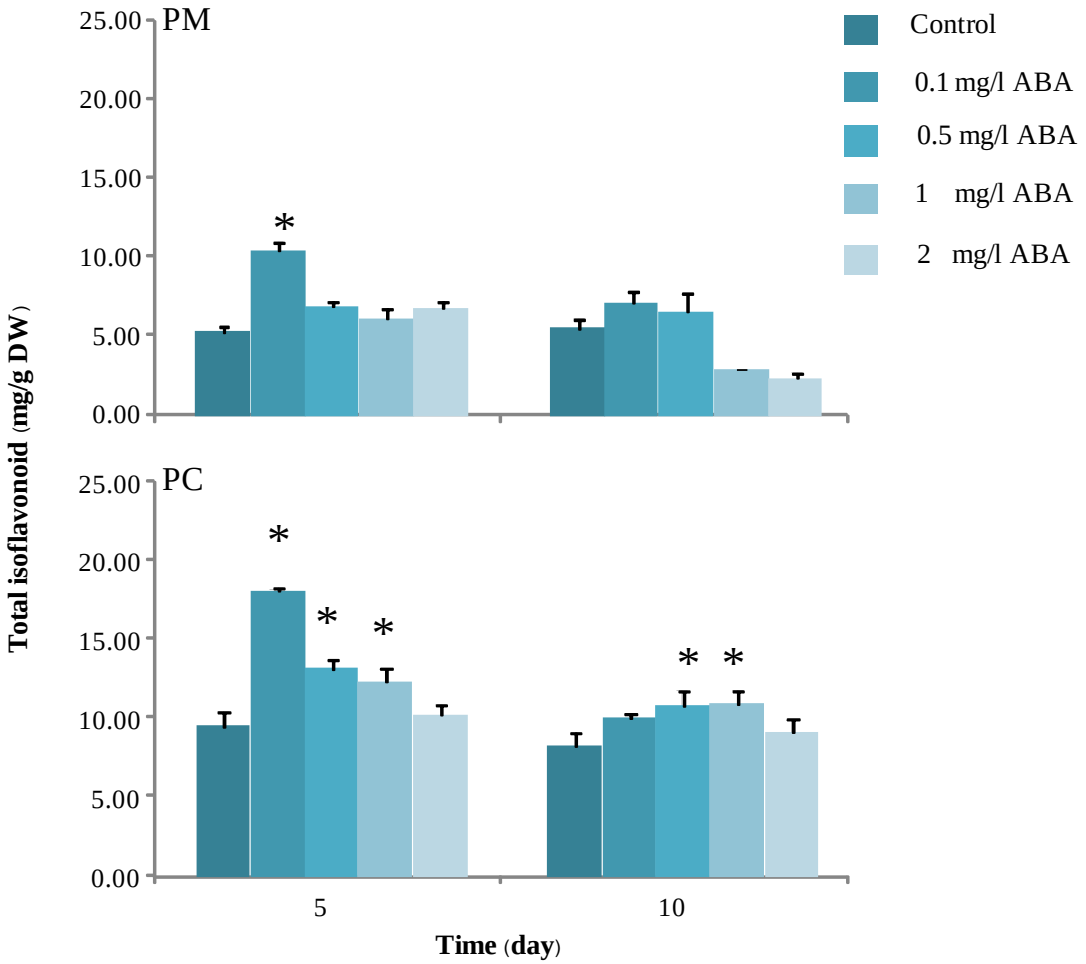


Figure 20 Effect of abscisic acids (ABA) on total isoflavonoid production in two varieties of *P. candollei* hairy root culture ($p \leq 0.01$)

2.5 Comparative analysis of hairy root and native root of both varieties of *P. candollei*

Comparative analysis of hairy root and native root of both varieties of *P. candollei* was investigated to prove chromene biosynthesis hypothesis that could occurred in cell with ability to produce chloroplast. The 7 weeks of hairy root and native roots of both *P. candollei* var. *mirifica* (PM) and *P. candollei* var. *candollei* (PC) from seeding were collected. Root samples were dried at 50°C in hot air oven to a constant weight for analysis of chromene and total isoflavonoid contents as shown in table 11. The comparative analysis of each phytoestrogen between hairy root and

native root for chromene content in PM hairy root, PC hairy root, PM root and PC root were 5.51±0.70, 4.62±0.15, 18.86±2.60 and 17.10±0.40 µg/g dry wt., respectively (Figure 21). Total isoflavonoid content in PM hairy root, PC hairy root, PM root and PC root were 7.72±0.43, 9.10±0.64, 6.64±0.46 and 7.05±0.82 mg/g dry wt., respectively.

Table 11 Comparative analysis of chromene and total isoflavonoid contents in hairy root and native root of both varieties of *P. candollei*

Type of roots	**Chromene content (µg/g dry wt)**	**Total isoflavonoid content (mg/g dry wt)**
Hairy root		
P. candollei var. *mirifica*	5.51±0.70	7.72±0.43
P. candollei var. *candollei*	4.62±0.15	9.10±0.64
Native root		
P. candollei var. *mirifica*	18.86±2.60	6.64±0.46
P. candollei var. *candollei*	17.10±0.40	7.05±0.82

Values represent the mean ± S.D. (n = 3)

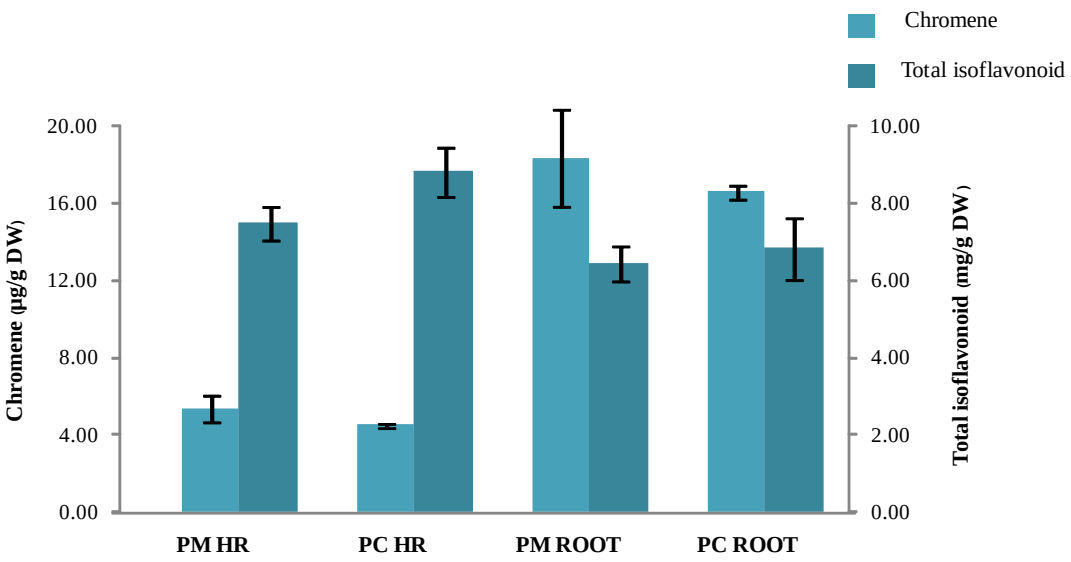


Figure 21 Comparative analysis of chromene and total isoflavonoid content in hairy root and native root

PM HR; *P. candollei* var. *mirifica* hairy root,

PC HR; *P. candollei* var. *candollei* hairy root,

PM ROOT; *P. candollei* var. *mirifica* native root,

PC ROOT; *P. candollei* var. *candollei* native root