CHAPTER V
DISCUSSION

1. Callus induction

P. candollei var. *mirifica* callus were derived from stem explants in MS medium supplemented with 0.1 mg/l TDZ, 1 mg/l NAA and 0.5 mg/l BA. The cell suspension characteristic was green, friable and rapid growth as previous report. Udomsuk et al. **(**2011**)** suggested that stem part was an optimal explant and combination of three hormones generated the highest yield of chromene and total isoflavonoid. According to Udomsuk et al. (2009), TDZ highly enhanced the production of isoflavonoid.
However stem-derived callus of *P. candollei* var. *mirifica* with only TDZ supplemented developed browning characteristics and cell growth decreased meanwhile stem-derived callus with TDZ in combination with NAA and/or BA was normally characteristic. The data from another study found the highest isoflavonoid produced by *P. candollei* var. *candollei* stem-derived callus and *P. candollei* var. *mirifica* root-derived (Boosnongcheep et al., 2010). Very diverse parts of a plant and plant species may effect on response. The appropriate type of explants, suitable culture medium and environmental conditions are important considerations for its development.

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

The growth pattern of *P. candollei* var. *mirifica* cell suspension culture gradually grew during the first week to reach the highest dry biomass at the last forth week (0.57±0.05 g/flask, dry wt) similar to previously report by Korsangruang et al. (2010) and Udomsuk et al. (2011). The pattern of chromene and total isoflavonoid accumulation in cell suspension at the 4^{th} week from our study decreased about 2 and 5-fold, respectively, compare with Udomsuk et al. (2011). This occurrence due to callus instability after several subculture in cycle of culture. The decreasing of plant secondary metabolite production could possibly relate to random spontaneous

variations during the culture process, which would be undesirable and limitation in the plant tissue culture system (Bairu et al., 2011).

3. Hairy root induction

Hairy root of *P. candollei* var. *mirifica* were fast growth and lateral branching as well as *P. candollei* var. *candollei* hairy root which was established by Udomsuk et al. (2009). *P. candollei* var. *mirifica* hairy root had hard fragile texture while *P. candollei* var. *candollei* had softened look like spongy texture. These results were similar to previously studied that found in hairy roots culture of *P. candollei* var. *mirifica* (Thanonkeo, 2006).

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

Hairy root slowly grow up during the first week to the forth week and reach to the highest biomass at the last forth week (0.11±0.02 g/flask, dry wt.) then the growth rate was decreased. The pattern of secondary metabolite accumulation in hairy root was similar to previously reported by Thanonkeo (2006) and Udomsuk et al. (2009). Hairy root generally produced both chromene 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 was chosen for further study.

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

Cell suspension culture and hairy root culture are general and valuable methods in plant tissue culture for enhance plant secondary metabolites by elicitation techniques. Various types of elicitors were introduced to cell suspension and hairy root culture; for example, biotic elicitors from fungi, bacteria, viruses, plant cell wall component and abiotic elicitors as inorganic compounds. In this study showed effect of elicitors

on chromene and total isoflavonoid production in both cell suspension and hairy root culture.

Cell suspension culture were treated with various types and concentrations of elicitors. Methyl jasmonate (50, 100 and 200 µM), yeast extract (0.5, 1.0 and 2.0 mg/ml) and chitosan (0.5, 1 and 10 mg/l) were added in culture medium for 3 and 6 days of elicitation. Our results indicate that some concentrations of methyl jasmonate, yeast extract and chitosan significantly increased chromene and total isoflavonoid production. However the level of total isoflavonoid accumulation in cell suspension culture was lower than previously reports about 5-fold (Korsangruang et al., 2010 and Udomsuk et al., 2011). The decreasing of secondary metabolite production due to callus instability after several subculture in cycle (Bairu et al. 2011). From our result showed 200 µM methyl jasmonate and 1 mg/ml yeast extract significantly increased highest chromene production after 3 days of elicitation (11.61±0.55, 7.84±0.53 µg/g dry wt, 2-fold higher than control). Chromene are prenylated compound which occurred via prenylation by prenyltransferase enzyme. Sasaki et al. (2008) found prenylated flavonoid in *S. flavescens* plant was induced by methyl jasmonate and salicylic acid. In similar study by Akashi et al. (2009), soybean cell culture with yeast treatment enhanced prenylated isoflavonoid production and induced prenyltransferase activity. According to our result showed chromene were elicited by methyl jasmonate, yeast and chitosan. These results indicate that chromene biosynthesis may be related to prenyltransferase.

Isoflavonoid are the major compound containing in *P. candollei* tuber root. This study indicates that 50 µM methyl jasmonate significantly increased total isoflavonoid production in cell suspension culture after 3 days of elicitation (4.40±0.29 mg/g dry wt, 2-fold higher than control). Yeast extract at 0.5 mg/ml for 6 days showed the highest enhancing of total isoflavonoid content (9.61±0.50 mg/g dry wt, 3-fold higher than control). We also found effect of chitosan at 0.5 mg/l could elicit highest both chromene and total isoflavonoid production after 6 days of elicitation (6.58±0.49 µg/g dry wt and 7.41±0.66 mg/g dry wt, respectively). When compare our result to other studies in *P. candollei* cell suspension culture, Thanonkeo (2006) showed methyl jasmonate could elicit daidzein and genistein higher than control group 6-fold, with the optimal duration 12 days of elicitation. Korsangruang et al. (2010) found 0.2 mM

methyl jasmonate could elicit the highest isoflavonoid production (40.5 mg/g dry wt). Yeast extract and chitosan also found to induce isoflavonoid in the same study.

For hairy root culture, the transformed roots were treated with 200 µM methyl jasmonate, 0.5 mg/ml yeast extract and 0.5 mg/l chitosan. The elicitor types and concentrations used in this study base on previous report for enhancement isoflavonoid production in hairy root culture of *P. candollei* var. *candollei* by Udomsuk et al. (2011). The results found 200 µM methyl jasmonate and 0.5 mg/ml yeast extract significantly increased chromene production after 3 days and 6 days of elicitation, respectively. While 200 µM methyl jasmonate and 0.5 mg/ml yeast extract highly significantly increased total isoflavonoid production after 3 days of elicitation (16.06±1.28 and 14.65±1.44 mg/g dry wt, 2-fold higher than control, respectively). Whereas study of Udomsuk et al. (2010) in *P. candollei* var. *candollei* hairy root culture found 200 µM methyl jasmonate and 0.5 mg/ml yeast extract highly significantly increased total isoflavonoid production after 6 days and 3 days of elicitation, respectively. The result 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. The production of both active compounds may decrease due to PM hairy root treated with chitosan developed browning during treatment. Previous study, Korsangruang et al. (2010) found high concentration of chitosan (50 mg/l) suppressed growth of PM and PC cell suspension culture. Although chitosan at high concentration (150 mg/l) was effective elicited total isoflavonoid in PC hairy root (Udomsuk et al., 2011) but from our observed in the preliminary study (data not shown) PM cell suspension treated with chitosan developed browning and some substance leaked into medium.

The variations of chromene and isoflavonoid production and treatment interval of elicitation may be due to variation of both *P. candollei* varieties, characteristic of plant cell and also culture condition.

Miroestrol and deoxymiroestrol are minor chromene compounds, which be found in *P. candollei* var. *mirifica* but act as highly estrogenic activity (Matsumura et al., 2005). However, only deoxymiroestrol, the highest estrogenic activity, can be found in our experiment. Due to hypothesis of chromene biosynthesis pathway, prenyltransferases is a key enzyme in prenylation reaction converting to chromene.

Accordingly studies reported on prenyltransferases activity which induced by methyl jasmonate and yeast extract (Sasaki et al., 2008 and Akashi et al., 2009). Our results found methyl jasmonate and yeast extract significantly increased chromene production in both cell suspension and hairy root culture. This data suggest that chromene biosynthesis possible to relate with prenyltransferase enzyme. In same studies (Sasaki et al., 2008 and Akashi et al., 2009) also found isoflavone synthase activity or IFS (the key enzyme in isoflavonoid biosynthesis pathway) was also induced by methyl jasmonate and yeast extract.

Although cell suspension culture technique was useful and potential for continually cultured and produced bioactive compounds. But its limitation was somaclonal variation after several subculture due to variation in plant secondary metabolite production as generally found in many studies (Deus-Neumann and Zenk, 1984 and Sengupta et al., 1988). For these reasons, hairy root culture was an alternative source because of its stability higher than cell suspension culture.

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

Plant hormones or phytohormones play a crucial role in controlling plants growth and development. There are five classes of phytohormones for instance, auxins, cytokinins, gibberellins, abscisic acid, and ethylene. The common characteristics of plant hormones are that they are natural compounds with an ability to affect physiological processes at very low concentrations. It is known that auxin-cytokinin interactions can regulate growth and organized development in plant tissue and organ cultures (Evans et al., 1981 and Vasil and Thorpe, 1994). In generally plant hormone were used in axillary or adventitious bud proliferation, root and callus formation. Otherwise, the application of plant hormone using as enhancer for the production of secondary metabolite in plant tissue culture were reported (Nagira et al., 2006; Angelova et al., 2006; Cristiane et al., 2009; Inthima et al., 2009; Gagne' et al., 2010 and Sun et al., 2011).

CPPU and TDZ are cytokinin plant growth regulator that useful for stimulate cell division and release of lateral bud dormancy. Our result showed addition various concentrations of CPPU and TDZ significantly increased the production of total

isoflavonoid in both PM and PC hairy root cultures after 5 days and 10 days of elicitation. Furthermore PM hairy root treated with various concentrations of TDZ showed effect of dose response when increasing TDZ-concentrations, the total isoflavonoid content was decreased after 5 days of elicitation. Our study is similar to previous reports on TDZ and CPPU to enhance flavonoid or terpenoid (Cristiane et al., 2009 and Inthima et al., 2009). Recently, production of isoflavonoid in callus culture of *P. candollei* var. *mirifica* (PM) was reported to be influenced by plant hormone, thiadiazuron (Udomsuk et al., 2010).

The plant hormone abscisic acid (ABA) plays a major role in seed maturation and germination, as well as in adaptation to abiotic environmental stresses. Various concentrations of ABA showed significantly increased the production of total isoflavonoid in PC hairy root culture with dose response as TDZ. Nonetheless, only of ABA 0.1 mg/l for 5 days significantly increased total isoflavonoid content in PM hairy root culture. The data from another publication were reported effect of ABA on enhancing flavonoid content in various plants (Nagira et al., 2006; Gagne' et al., 2010 and Lacampagne et al., 2010).

As a result, PM hairy root was responded to cytokinin greater than PC hairy root. In contrast, abscisic acid was more influence to PC hairy root than the other one. Indicate that plant varieties may affect on elicitor responded due to their genetic variation and chemical component containing (Cherdshewasard et al., 2007; Boonsnongcheep et al., 2010; Korsangruang et al., 2010 and Yusakul et al., 2011).

In summarize, plant hormone response might alter the production of total isoflavonoid associated with defense mechanisms in the plant or the hormonal pathway. The ABA biosynthesis pathway has enabled to be elucidated in higher plants (Taylor, 1991; Schwartz et al., 1997; Zeevaart and Creelman, 1988). Based on above results, 5 days of elicitation was an appropriate incubation time with low concentration of hormones to enhance both bioactive compound productions in two varieties of hairy root cultures. The plant cell may take longer incubation time to contact with elicitor substance due to hormone response time in plant cell.

Finally, in cell suspension culture study we did not use plant hormone as elicitors because of their culturing already had hormone supplement to maintain the callus formation

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

Concerning the hypothesis of chromene biosynthesis pathway, it is possible that the chromene skeleton can be derived from enzymatic prenylation reaction of isoflavan and dimethylallyl pyrophosphate (DMAPP) (Yu and McGonigle, 2005; Dewick, 2009). The prenylating enzymes are generally localized to plastid, where the reaction was occurred. Therefore, chromene biosynthesis may take place in chloroplast cell. To prove chromene biosynthesis hypothesis that could occurred in cell with ability to produce chloroplast, thus we comparative analysis of hairy root and native root of both varieties of *P. candollei*. The comparative analysis of each phytoestrogen between hairy root and native root found that chromene content in PM hairy root, PC hairy root, PM root and PC root were 5.51, 4.62, 18.86 and 17.10 µg/g dry wt, respectively. Total isoflavonoid content in PM hairy root, PC hairy root, PM root and PC root were 7.72, 9.10, 6.64 and 7.05 mg/g dry wt, respectively. Our results show biosynthesis of both compounds in hairy root when decreasing of chromene content, isoflavonoid content was also increased. In contrast chromene content was increased; isoflavonoid content was decreased in native root. These suggest that chromene and isoflavonoid biosynthesis pathway may be related. In chromene biosynthesis pathway, the prenylation reaction needs isoflavan which was the substrate derived from isoflavonoid biosynthesis pathway. These resulting in decreasing of substrate that was turn to isoflavonoid then seeing the biosynthesis alteration of two compounds content in this experiment. The levels of isoflavonoid content were not significantly difference between hairy root and native root. This indicated that isoflavonoid biosynthesis can be occurred and accumulated in root part. Whereas higher chromene accumulation in native roots than hairy roots, it is possible that its biosynthesis may take place in plastid of leaves part then being localized and accumulated to the root part. In addition, the structures of chromene are more complex than those of isoflavonoid and chromene biosynthesis pathway still doubt. However, to confirm these findings we need further study in-depth.