Plant Cell Tiss Organ Cult (2010) 103:333–342
DOI 10.1007/s11240-010-9785-6

ORIGINAL PAPER

Effects of abiotic and biotic elicitors on growth and isoflavonoid accumulation in *Pueraria candollei* var. *candollei* and *P. candollei* var. *mirifica* cell suspension cultures

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Received: 1 May 2010 / Accepted: 15 June 2010 / Published online: 1 July 2010

Abstract This study demonstrates the effects of various concentrations of abiotic and biotic elicitors on the cell growth and isoflavonoid accumulation of *P. candollei* var. *mirifica* (PM) and *P. candollei* var. *candollei* (PC) cell suspension cultures. The two plant varieties exhibited different growth responses and varied isoflavonoid accumulation after the addition of elicitors. Copper sulfate, methyl jasmonate (MeJA), and yeast extract did not significantly affect the growth of either plant variety, whereas oligosaccharide and the biotic elicitors used in this study [i.e., 50 mg l^{-1} chitosan and all concentrations of laminarin (LAM)] suppressed the growth of PM. The addition of MeJA to the medium principally induced an effect on the isoflavonoid content in both PM and PC, with 2.0 μM MeJA inducing the highest isoflavonoid content, as indicated by the induction index—4.41 in PM and 9.62 in PC cells on the 12th and ninth day of culture, respectively. A maximum total isoflavonoid content of 40.49 mg g^{-1} dry weight was achieved in PM 21 days after elicitation with 2.0 μM MeJA. LAM elicited the PM cell suspension culture to produce puerarin, which was not found in the unelicited culture. The results of this study provide information that will be useful for enhancing the accumulation of isoflavonoids in *P. candollei* cell suspension cultures.

Keywords Elicitor · Fabaceae · HPLC · Isoflavonoid · Medicinal plant · *Pueraria candollei* · Tissue culture

Abbreviations

BA	6-Benzyladenine
CHI	Chitosan
$CuSO_4$	Copper sulfate
2,4-D	2,4-Dichlorophenoxyacetic acid
II index	Isoflavonoid induction index
JA	Jasmonic acid
LAM	Laminarin
MeJA	Methyl jasmonate
PC	*P. candollei* var. *candollei*
PM	*P. candollei* var. *mirifica*
YE	Yeast extract

Introduction

Pueraria candollei var. *candollei* (PC) and *P. candollei* var. *mirifica* (PM) (Fabaceae) are two well-known legume species. In Thailand, they are commonly known as "kwao krua". The botanical characteristics of both plants are similar; however, PM has shorter pods covered with longer hairs (Prathanturarug et al. 2000; van der MaeSen 2002). The dried, tuberous roots of PM are used ethnomedically as a tonic for rejuvenation in elderly individuals (Kashemsanta and Lakshnakara 1952). The chemical constituents of these plants include chromenes, coumestrans, and

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isoflavones, such as daidzein, genistein (and their glycosides, daidzin and genistin), and puerarin, all of which possess estrogenic activity (Chansakaow et al. 2000; Ingham et al. 1986; Tahara 1987). *P. candollei* is currently used as an active ingredient in numerous herbal and cosmetic products.

Plants have many defensive systems that work as protective mechanisms against disturbance or attack by physical, chemical, microorganismal, or biological factors. After triggering the signal transductions of the defensive pathway, transcription factors regulate the production of secondary metabolites by means of phytoalexins—low-molecular-weight substances with various useful biological actions (Endress 1994; Evans 2002; Zhao et al. 2005). The production, accumulation, and excretion of secondary metabolites into cell cultures can be induced by both biotic and abiotic elicitors (Braz et al. 1990; Endress 1994). The abiotic elicitation effects of heavy metals, in the form of inorganic salts added to the culture medium, have studied in plant culture systems. For example, copper (Cu) is usually used in the form of sulfate or chloride salts to induce secondary metabolite production in plants (Bhuiyan and Adachi 2003; Engelmann et al. 2009; Hakamatsuka et al. 1991; Liu and Dixon 2001). The jasmonic acid (JA) signaling pathway is a transducer or mediator for elicitor signaling that can lead to the accumulation of plant secondary metabolites (Zhao et al. 2005). Exogenous applications of jasmonic acid and its related compounds (such as methyl jasmonate, MeJA) have been used to study the effects of these elicitors on plant secondary metabolite production (Chen and Chen 2000; Gadzovska et al. 2007; Hayashi et al. 2003; Kirakosyan et al. 2006; Modolo et al. 2002). Biotic elicitors have been intensively studied with respect to their role in isoflavonoid accumulation via various mechanisms of elicitation. Yeast extract (YE) is often used as a biotic elicitor in plant–microbe interaction studies. For example, YE was used to induce chalcone synthase (*CHS*) expression in cell suspension cultures of *P. lobata* to clone the gene-coding enzyme (Nakajima et al. 1991). Poly- or oligosaccharides are signaling molecules with elicitation pathways that have been intensively studied because these compounds can substitute for fungal elicitors during a pathogen attack (Zhao et al. 2005). Chitin and chitosan (CHI, deacetylated chitin) are exogenous biotic elicitors that are derived from the fungal cell wall (Montesano et al. 2003). These elicitors have been studied for their effects on phenylpropanoid metabolic enzymes (Funk and Brodelius 1990) and secondary metabolite production (Zhang et al. 2000, Wise et al. 2009). Laminarin, a linear β-1,3-glucan, is an interesting glucan elicitor that has also been used in studies on the effects of elicitation on secondary metabolite production (Aziz et al. 2003; Cosi et al. 2007; Klarzynski et al. 2000).

Plant cell cultures are appropriate tools for examining secondary metabolite production (Evans 2002), especially cell suspension culture techniques that can aid in the understanding of elicitation and immobilization, including how these processes function in bioreactor studies (Bourgaud et al. 2001; George 2008). Cell suspension cultures from PC and PM have recently been established to concomitantly analyze isoflavonoid compounds (Boonsnongcheep et al. 2010). There are a number of publications related to the improvement of isoflavonoid accumulation using various elicitors in cell cultures of *Pueraria* spp. (Goyal and Ramawat 2008; Hakamatsuka et al. 1991; Maojun et al. 2006; Park et al. 1995). However, to the best of our knowledge, there have been no reports on the effects of elicitors on isoflavonoid accumulation in *P. candollei* cell suspension cultures.

The aims of this study were (1) to determine the effects of various concentrations of abiotic and biotic elicitors on the cellular growth rate and the accumulation of isoflavonoids in *P. candollei* cell suspension cultures and (2) to identify the most effective elicitor capable of inducing the highest amount of isoflavonoids. Elicitors were applied in the pre-exponential phase of the growth curve (representing the period when the cell is preparing for growth) in order to study their effect on the growth promotion that is associated with secondary metabolism. The data derived from this study will be useful in improving isoflavonoid production in *P. candollei* cell suspension cultures in various fields of plant biotechnology and will also aid in the study of the isoflavonoid biosynthesis pathway.

Materials and methods

Plant cell cultures and elicitor treatments

Calli were obtained from PM and PC roots using the protocol established by Boonsnongcheep et al. (2010). A 500-mg sample of callus tissue was transferred into a 125-ml Erlenmeyer flask containing 50 ml of MS (Murashige and Skoog 1962) medium supplemented with 0.56 μM 6-benzyladenine (BA) and 4.52 μM 2,4-dichlorophenoxyacetic acid (2,4-D). The cultures were maintained on a shaker at 110 rpm at 25 ± 3°C under a 16/8-h light/dark photoperiod with light supplied at an intensity of 37.5 $\mu Em^{-2}\ s^{-1}$. Six days after initiation of the cell suspension culture, an aliquot of each elicitor [copper (II) sulfate ($CuSO_4 \cdot 5H_2O$) (Merck, Whitehouse Station, NJ), MeJA (Sigma–Aldrich, St. Louis, MO), YE (Merck), CHI (minimum 85% deacetylated, Sigma), and laminarin (LAM) (Sigma–Aldrich)] was added to separate flasks. The final concentrations of each elicitor were (1) $CuSO_4$: 0.125, 0.625, 1.25 mg l^{-1} (equivalent to 5-, 25- and 50-fold $CuSO_4$ in

MS media, respectively); (2) MeJA: 0.5, 1.0, 2.0 μM; (3) YE: 0.5, 1.0, 2.0 mg l^{-1}; (4) CHI: 1.0, 10.0, 50.0 mg l^{-1}; (5) LAM: 0.05, 0.1 0.2 mg ml^{-1}. The cells were incubated with each elicitor for a further 21 days under identical growth conditions.

The controls for the $CuSO_4$, YE, and LAM treatments contained sterile deionized water in the same quantities as the added elicitors. The controls for the CHI and MeJA treatments contained 1% acetic acid (Merck) and absolute ethanol (Merck) solvent, respectively, which were added in the same quantities as the elicitors. The pH of all of the experimental media was adjusted to 5.8 before autoclaving. Stock $CuSO_4$, YE, LAM, and CHI solutions, including the 1% acetic acid solution, were sterilized by autoclaving, whereas the stock MeJA solution and ethanol were filter-sterilized through a 0.20-μm syringe filter.

Cell growth and isoflavonoid content determination

Following the addition of the elicitor to the cell suspension, three flasks for each treatment, including the control group, were collected every 3 days from the ninth to the 27th day of culture. The collected cells were dried at 60°C for 48 h to obtain the dried biomass as described by Boonsnongcheep et al. (2010). The growth index for the *P. candollei* cell suspension cultures was determined on a phytomass dry weight (DW) basis as follows: dry growth index = (Final DW − Initial DW)/Initial DW (Kittipongpatana et al. 1998).

For the isoflavonoid analysis, each sample was extracted three times with methanol (anhydrous; JT Baker, Deventer, the Netherlands) using a sonication technique at 45°C for 30 min. After complete evaporation, 100 mg of the extract was dissolved with methanol [high-performance liquid chromatography (HPLC) grade; Fisher Chemical, Thermo Fisher, Waltham, MA], and the volume was adjusted to 10.0 ml for HPLC analysis. Separation was performed in a purospher RP-18 column (250 × 3.0 mm i.d., particle size 5 μm). The gradient of the mobile phases between solvent A (0.2% acetic acid in water) and solvent B (acetonitrile) was carried out using validated simultaneous HPLC (Boonsnongcheep et al. 2010).

To compare the induction effect of each elicitor on the isoflavonoid accumulation of the *P. candollei* cell suspension cultures, the isoflavonoid induction index was determined from the total isoflavonoid content using the following equation: isoflavonoid induction index (II Index) = (Total $\text{isoflavonoid}_{\text{elicitor}}$ − Total $\text{isoflavonoid}_{\text{control}}$)/Total $\text{isoflavonoid}_{\text{control}}$.

Statistical analysis

Three replications were conducted for each treatment. The data obtained were analyzed using analysis of variance (ANOVA) and Duncan's analysis with significance set at a p value = 0.05.

Results and discussion

Growth and isoflavonoid accumulation of the untreated *P. candollei* cell suspension cultures

Figure 1 shows the growth pattern of the untreated cell suspension cultures (control) based on dried biomass. The stationary phase of the growth curve indicates that PM cell suspension cultures grew about 1.5-fold better than those of PC. The highest average dried biomass from all of the experiments of the untreated PM phytomass was 490.27 mg $flask^{-1}$, which occurred on the 24th day of culture; the untreated PC cell suspension cultures had an average dried biomass of 345.99 mg $flask^{-1}$ on the 27th day. The cell suspension cultures of both varieties reached the stationary phase at the 24th day of cell culture (Fig. 1), as indicated by the growth index. Daidzin and genistin were the major glycoside compounds present in the culture medium of both varieties. The highest average total isoflavonoid contents in the PM and PC cell suspension cultures were found on day 27 of the culture and measured 21.67 and 19.58 mg g^{-1} DW, respectively. The ratios of non-conjugated:conjugated isoflavonoid (daidzin:daidzein and genistin:genistein) accumulation in the PM cell suspension were 16:9 and 3:1, respectively, whereas the PC cell suspension accumulated these isoflavonoids at ratios of 3:1 and 7:1, respectively. Our results show the same pattern of isoflavonoid accumulation in *P. candollei* cell suspension cultures as was previous reported by Boonsnongcheep et al. (2010).

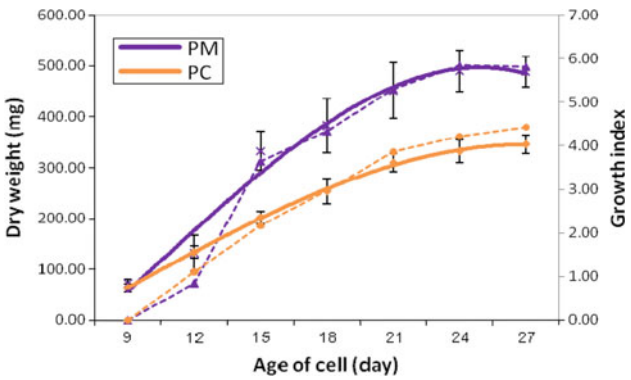

Fig. 1 Growth and growth index of the *Pueraria candollei* cell suspension cultures (*PC Pueraria candollei* var. *candollei*, *PM P. candollei* var. *mirifica*). The *lines* show the average dried biomass of the untreated cells from all experiments. *Solid line* dry weight, *dashed line* growth index

The effects of elicitors on the growth of *P. candollei* cell suspension cultures

The growth of PM and PC cell suspension cultures was not affected by the addition of $CuSO_4$, MeJA, and YE to the medium at the concentrations used in this study (Fig. 2a, b). High concentrations of CHI (50 mg l^{-1}) significantly suppressed the growth of PM (from the 12th day to the 27th day of culture) and PC (from the 15th day to the 18th day of culture) ($p = 0.05$). LAM, at all of the concentrations examined, also significantly suppressed the growth of PM up to the 12th day of culture ($p = 0.05$); however, it had no significant effect on the growth of the PC cell suspension (Fig. 2). The LAM treatments at 0.1 and 0.2 mg ml^{-1} slightly decreased the dried biomass of the PC cells between the 15th day and the 18th day of the culture. In addition to the growth-suppression effects of CHI and LAM, cells treated with these compounds also developed serious browning. The browned cells could, however, slowly re-grow within the phase of exponential growth. These results demonstrate that the oligosaccharide biotic elicitors (CHI and LAM) are able to suppress the growth of *P. candollei* cell suspension cultures, especially in PM cells, while $CuSO_4$, MeJA, and YE did not significantly affect the growth of the suspension cultures.

The effects of $CuSO_4$ on isoflavonoid accumulation

After the PM and PC cell suspension cultures were treated with $CuSO_4$, a slight accumulation effect in the total isoflavonoid content was observed until the cells reached a stationary phase (Fig. 3). When PM cells were treated with five doses of $CuSO_4$ in MS media, the II Index gradually increased from the 12th day (0.21) to the 18th day (1.24). In contrast, in the PC cell culture, the highest II Index (2.04) was obtained 3 days following the addition of $CuSO_4$ (five doses) to MS media (Fig. 8a). Because polyphenolic compounds, such as isoflavonoids, act as metal-chelating compounds that could alleviate metal toxicity in plant cells (Jung et al. 2003), it is possible that the five-fold treatment dosage of $CuSO_4$ in this study induces the accumulation of isoflavonoids in PM cells.

Plant cells generate hydrogen peroxide (H_2O_2) after receiving a signal from abiotic elicitors such as metal ions (Apel and Hirt 2004). In general, high doses of metal, such as Cu, may cause toxicity in cells by interfering with enzymatic activity, causing DNA alterations, disturbing metabolism, and inhibiting growth (Fernandes and Henriques 1991; Maksymiec 1997). Ali et al. (2006) reported that the concentration and the time of Cu exposure affected the growth of *Panax ginseng* root cultures, including the ginsenoside content and other enzyme activities, and that Cu uptake also increased in the cells. Our results may imply that $CuSO_4$, especially at higher concentrations (25- and 50-fold doses in the MS media), affects *P. candollei* cells after long periods of exposure and causes a decrease in isoflavonoid accumulation when cells were closed to the stationary phase of their growth.

The effects of MeJA on isoflavonoid accumulation

Methyl jasmonate, a well-known abiotic elicitor, induced isoflavonoids in the PM and PC cell cultures at all concentrations. The addition of MeJA generally increased the total isoflavonoid content after 3 days of elicitation (i.e., on day 9 of culture) (Fig. 4). The addition of 1.0 and 2.0 μM MeJA significantly increased the total isoflavonoid content in the PM cells to a higher level than the control from the 12th day to the 21st day of culture ($p = 0.05$), whereas 2.0 μM MeJA significantly enhanced the total isoflavonoid content in the PC cell suspension from the ninth day to the 18th day of culture ($p = 0.05$). MeJA (2.0 μM) enhanced the accumulation of isoflavonoids; the II Index was 4.41 in PM cells on the 12th day and 9.62 in the PC cells on the ninth day of culture (Fig. 8b). These results demonstrate that MeJA induced isoflavonoid accumulation in the early growth phase after the addition of the elicitor in both plant varieties. Comparable trends for the early induction of MeJA were observed for anthraquinones, anthocyanin, and stilbene in their accumulation in morinda, ohelo, and grape cell suspension cultures, respectively (Chong et al. 2005; Fang et al. 1999; Krisa et al. 1999).

In previous studies, a high concentration of MeJA was found to inhibit the growth of plant cell cultures (Bhuiyan and Adachi 2003; Colque et al. 2004; Zhao et al. 2010). Our preliminary study found that MeJA at concentrations of 5, 50, and 100 μM almost caused cell death after 3–6 days of elicitation (data not shown). Given these results, the concentrations of MeJA that were used in this study were not harmful to the cell growth and were accompanied by the enhancement of isoflavonoids and their glycoside accumulation.

The effects of YE on isoflavonoid accumulation

In this study, YE enhanced the early accumulation of isoflavonoids after elicitation. Treatments with 0.5, 1.0, and 2.0 mg l^{-1} YE resulted in II Indices of >0.5 on the ninth and 12th day in PM cell cultures and on the ninth day in PC cell cultures. The II Index approached negative values or displayed a suppression effect on the 15th day of culture, except in the PM cells that were treated with 2.0 mg l^{-1} YE (Fig. 8c). Daidzein and genistein were induced by all concentrations of YE by day 12 following the addition of the elicitor to the medium (18th day of culture), whereas the non-glucoconjugated compounds were found at lower

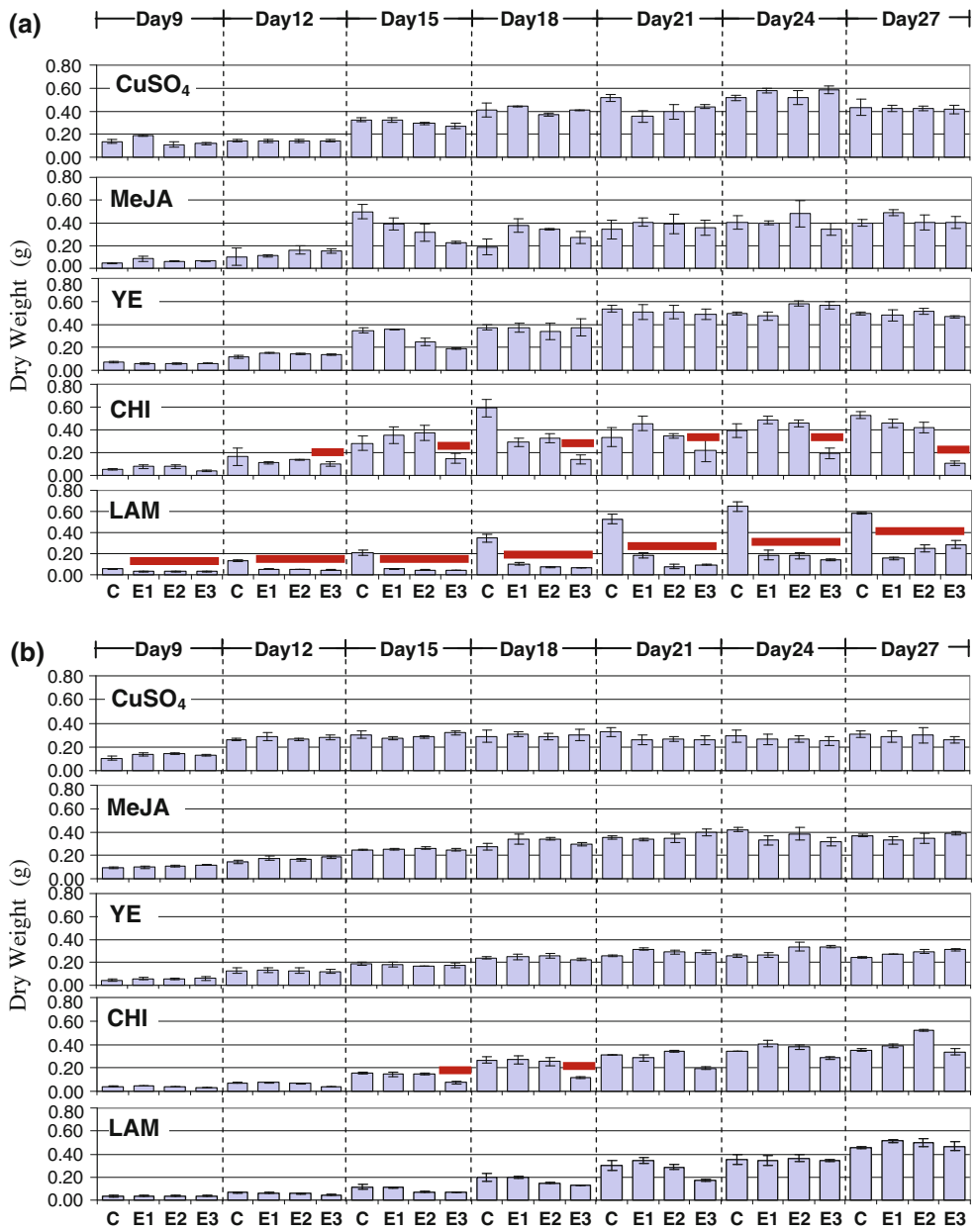

Fig. 2 Growth of *P. candollei* var. *mirifica* (**a**) and *P. candollei* var. *candollei* (**b**) cell suspension cultures after elicitation. The cells were treated with copper sulphate (*$CuSO_4$*; *C* Control, *E1* 0.125 mg l^{-1}, *E2* 0.625 mg l^{-1}, *E3* 1.25 mg l^{-1}), methyl jasmonate (*MeJA*; *C* control, *E1* 0.5 μM, *E2* 1.0 μM, *E3* 2.0 μM), yeast extract (*YE*; *C* control, *E1* 0.5 mg l^{-1}, *E2* 1.0 mg l^{-1}; E3 = 2.0 mg l^{-1}), chitosan (*CHI*; *C* control, *E1* 1.0 mg l^{-1}, *E2* 10.0 mg l^{-1}, *E3* 50.0 mg l^{-1}), and laminarin (*LAM*; *C* control, *E1* 0.05 mg l^{-1}, *E2* 0.1 mg l^{-1}, *E3* 0.2 mg l^{-1}). The age of the cells is indicated by the day and is partitioned by *dashed lines*. The *bold lines* show the suppression effect of elicitors on the growth of cells when compared with the control at the 95% confidence interval (95% CI)

concentrations in the control cultures of both cell varieties (Fig. 5). Similar evidence of the increasing ratio between the isoflavonoid aglycones and their glucoconjugated compounds was also observed in the YE-treated roots of white lupin seedlings (Gagnon and Ibrahim 1997) and in the YE-treated cell suspension cultures of chickpeas (Mackenbrock et al. 1993). In our study, YE had a weak induction effect towards isoflavonoid accumulation in the PM and PC cell suspension cultures. Similarly, a number of publications report that YE is less effective towards plant cells than the other elicitors (Funk and Brodelius 1990; Gagnon and Ibrahim 1997; Sanchez-Sampedro et al. 2005).

The effects of CHI on isoflavonoid accumulation

In PM and PC cell suspension cultures, higher (50.0 mg l^{-1}) concentrations of CHI (β-1,4-linked glucosamine) decreased the total isoflavonoid content, whereas

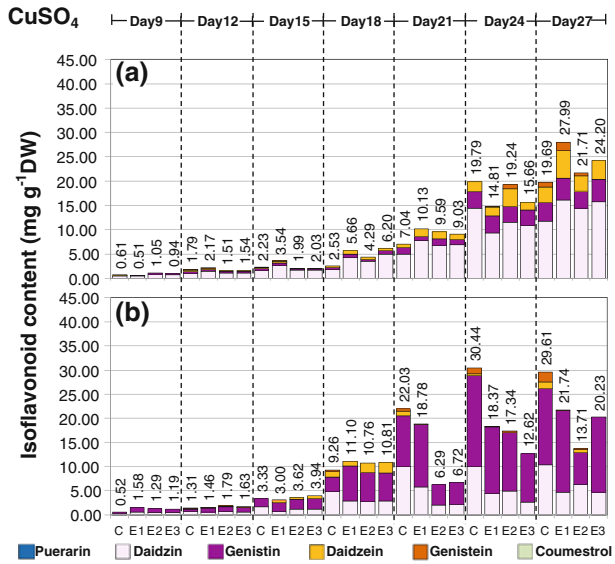

Fig. 3 Isoflavonoid profiles of *P. candollei* var. *mirifica* (**a**) and *P. candollei* var. *candollei* (**b**) cell suspension cultures after treatment with $CuSO_4$. *C* control, *E1* 0.125 mg l^{-1}, *E2* 0.625 mg l^{-1}, *E3* 1.25 mg l^{-1} (5-, 25-, 50-fold $CuSO_4$ in MS media, respectively). The age of the cells is indicated by the day, which is partitioned by *dashed lines*

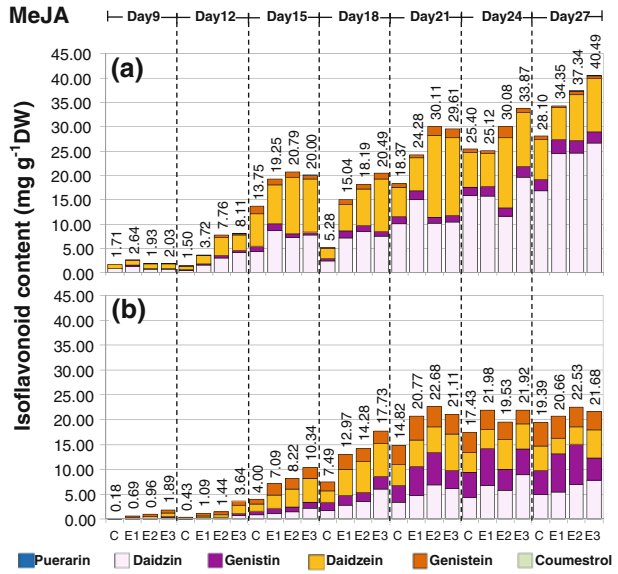

Fig. 4 Isoflavonoid profiles of *P. candollei* var. *mirifica* (**a**) and *P. candollei* var. *candollei* (**b**) cell suspension cultures after treatment with MeJA. *C* Control, *E1* 0.5 μM, *E2* 1.0 μM, *E3* 2.0 μM. The age of the cells is indicated by the day, which is partitioned by *dashed lines*

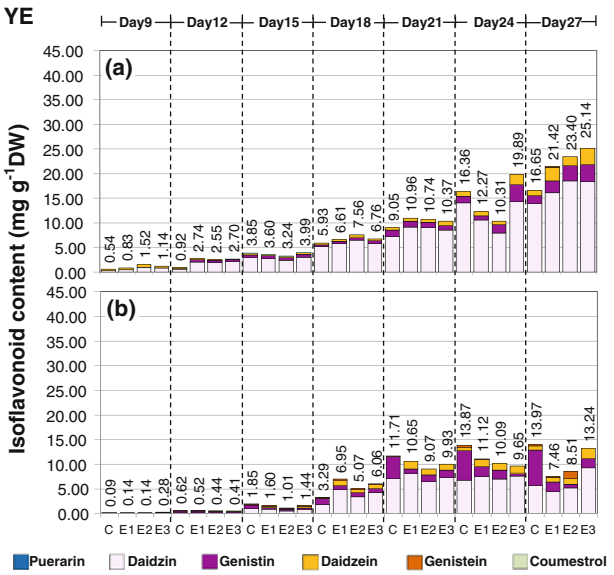

Fig. 5 Isoflavonoid profiles of *P. candollei* var. *mirifica* (**a**) and *P. candollei* var. *candollei* (**b**) cell suspension cultures after treatment with YE. *C* Control, *E1* 0.5 mg l^{-1}, *E2* 1.0 mg l^{-1}, *E3* 2.0 mg l^{-1}. The age of the cells is indicated by the day, which is partitioned by *dashed lines*

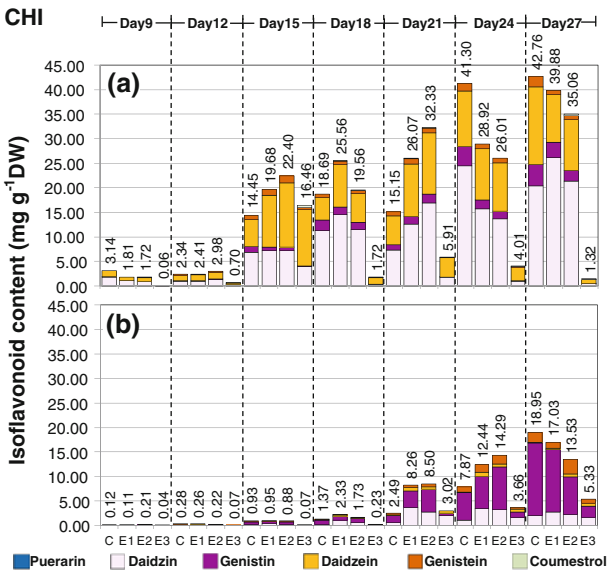

Fig. 6 Isoflavonoid profiles of *P. candollei* var. *mirifica* (**a**) and *P. candollei* var. *candollei* (**b**) cell suspension cultures after treatment with CHI. *C* Control, *E1* 1.0 mg l^{-1}, *E2* 10.0 mg l^{-1}, *E3* 50.0 mg l^{-1}. The age of the cells is indicated by the day, which is partitioned by *dashed lines*

concentrations ranging from 1.0 to 10.0 mg l^{-1} increased the total isoflavonoid content in both cell varieties (Fig. 6). CHI at 10.0 mg l^{-1} induced the highest II Index on the 21st day of culture, with II Index values of 1.13 and 2.41 in PM and PC cells, respectively (Fig. 8d). However, after both varieties arrived at the stationary phase of growth, a higher total isoflavonoid content was found in the controls relative to the CHI-treated cells. There have been reports of CHI being less effective than other biotic elicitors (Bhuiyan and Adachi 2003; Fang et al. 1999).

Coumestrol was detected in some samples of PM cells that were treated with 1.0, 10.0, and 50.0 mg l^{-1} CHI.

A relatively high amount of coumestrol (0.46 mg g^{-1} DW) was found in PM cells treated with 50 mg l^{-1} CHI on the 15th day of culture. It should be noted that a high concentration of CHI made the cells significantly brown with aging. The accumulation of coumestrol may relate to the browning of the cells, especially in the PM cells, which are more sensitive to stress than the PC cells. It is generally accepted that the production of compounds may relate to the stress level of the cell cultures, including the effectiveness of each elicitor (Vasconsuelo and Boland 2007). Coumestrol, a coumestans compound, has been reported to be found in the intact tuberous root of PM (Chansakaow et al. 2000; Ingham et al. 1988). In addition, Bourgaud et al. (1999) demonstrated that CHI increased the accumulation of coumestrol in the older (brown) tissue of *Psoralea lachnostachys* hairy root cultures, whereas daidzein was induced in younger tissue. In our study, the accumulation of coumestrol in the PM cell cultures may be related to stress-induced aging after the treatment with CHI.

The effects of LAM on isoflavonoid accumulation

LAM at 0.05 mg ml^{-1} significantly enhanced the production of isoflavonoid compounds in the PM cell suspension cultures on the 21st day ($p = 0.05$), whereas higher concentrations showed only a slight effect on isoflavonoid production (Fig. 7). The addition of 0.05 mg ml^{-1} LAM produced the highest isoflavonoid accumulation in PM cells and resulted in an II Index of 13.06 on the 27th day of culture (Fig 8e). The use of 0.05–2.0 mg ml^{-1} LAM caused browning in the PM cells that was similar to that observed in the CHI experiment. Although the cells were capable of re-growth after the 18th day of culture, the growth was still significantly suppressed from the day of elicitation to the stationary phase. This growth suppression may have been caused by the accumulation of phytoalexin compounds, which are biosynthesized in cells after a stress reaction. Some elicitors suppress primary metabolism and cause a shift towards secondary metabolite products that cause plant growth to slow down (Chong et al. 2005). Similar results for secondary metabolites associated with growth suppression were found in elicitor treatments on morinda, vanilla, and salvia cell suspension cultures (Chong et al. 2005; Funk and Brodelius 1990; Zhao et al. 2010). The PC cells were less sensitive to elicitors than the PM cell suspension cultures. LAM was not harmful to PC cells at any concentration and did not affect cell growth. Nevertheless, LAM did show a suppression effect in the PC cell suspension cultures after the 24th day of culture because it could not enhance the accumulation of isoflavonoid compounds as it did in the PM cells. LAM (1.0 mg ml^{-1}) has been reported to have a suppressive effect on resveratrol production in peanut hairy root cultures (Medina-Bolivar et al. 2007).

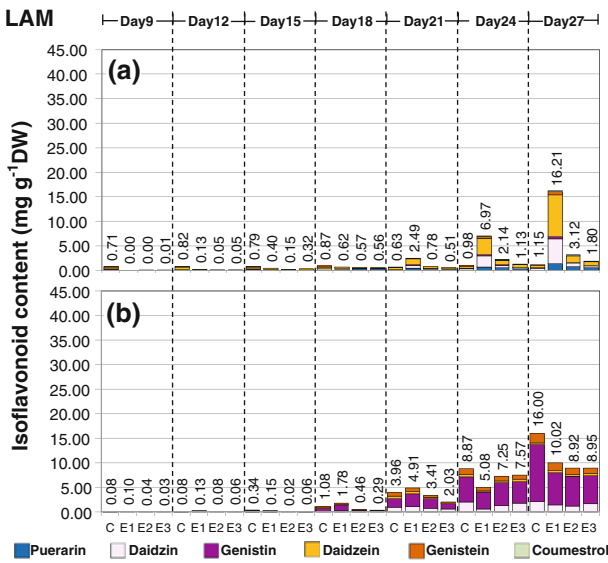

Fig. 7 Isoflavonoid profiles of *P. candollei* var. *mirifica* (**a**) and *P. candollei* var. *candollei* (**b**) cell suspension cultures after treatment with LAM. *C* Control, *E1* 0.05 mg ml^{-1}, *E2* 0.1 mg ml^{-1}, *E3* 0.2 mg ml^{-1}. The age of the cells is indicated by the day, which is partitioned by *dashed lines*

No puerarin was detected in the control groups of either plant variety, and this result is consistent with the findings of previous studies (Boonsnongcheep et al. 2010). It is noteworthy, however, that LAM elicited the production of puerarin in the PM cell suspension culture 15 days after the addition of the elicitor (Table 1). This finding was coincident with the very low amounts or the absence of genistin and genistein in the cell cultures that were treated with LAM (Fig. 7). This effect may be related to the biosynthesis of puerarin, which is a daidzein-8-*C*-glucoside. Daidzein and genistein are biosynthesized in the same pathway, but there is an intermediate compound that is changed at a branch point by a number of catalytic enzymes (Yu and McGonigle 2005). There are only limited data on the puerarin biosynthesis pathway (Inoue and Fujita 1974, 1977); LAM treatment may thus be used to study the expression of some genes that are involved in the puerarin biosynthesis pathways, especially in cell suspension cultures. LAM at 0.05 and 1.0 mg ml^{-1} also induced low amounts of coumestrol (<0.1 mg g^{-1} DW) in some samples of PM cell cultures when the cells reached the stationary growth phase (after the 24th day of culture) (Fig. 7).

Conclusion

Our results suggest that MeJA, the most-studied elicitor, had a superior induction effect on isoflavonoid accumulation

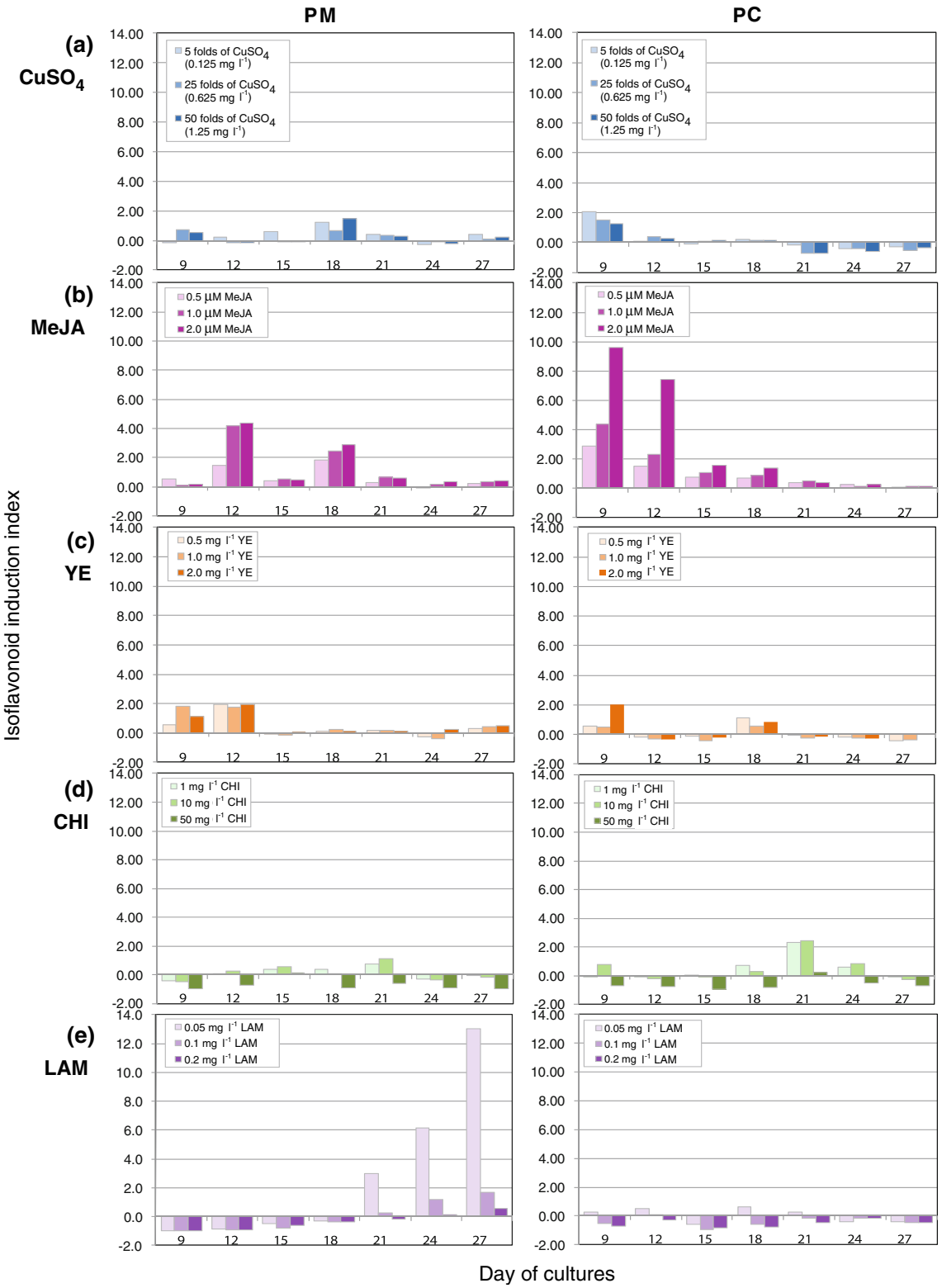

Fig. 8 Comparison of the induction effect of the elicitors on isoflavonoid accumulation in *P. candollei* var. *mirifica* (*PM*) and *P. candollei* var. *candollei* (*PC*) cell suspension cultures. The data are presented as the isoflavonoid induction index (II Index)

in both of the varieties of *P. candollei* cell suspension cultures studied here. After treatment with 2.0 µM MeJA, the total isoflavonoid contents in the PM cell suspension were the highest, 40.49 mg g^{-1} DW, on the 27th day compared with those found in the normal cell suspension cultures of PM, PC (Boonsnongcheep et al. 2010), and in

Table 1 Average puerarin contents in *Pueraria candollei* var. *mirifica* after elicitation with laminarin

Laminarin concentration (mg ml^{-1})	Day of culture (day after elicitation)						
	9 (3)	12 (6)	15 (9)	18 (12)	21 (15)	24 (18)	27 (21)
0.00	ND	ND	ND	ND	ND	ND	ND
0.05	ND	ND	ND	0.2244 ± 0.0115	0.3940 ± 0.0620	0.6890 ± 0.4597	1.3808 ± 0.4133
0.10	ND	ND	ND	0.2789 ± 0.0351	0.2345 ± 0.0560	0.5325 ± 0.1562	0.7657 ± 0.1662
0.20	ND	ND	ND	0.3107 ± 0.0433	0.1990 ± 0.0175	0.2562 ± 0.0249	0.5338 ± 0.0346

ND Not determined

Puerarin content is given in milligrams per gram dry weight

the elicitor-treated cell suspension of *P. tuberosa* (Goyal and Ramawat 2008) from previous studies. These results may imply that MeJA is connected directly to jasmonate signaling, including the oxylipin biosynthesis pathway, whereas the biotic elicitors have more complex stress responses. None of the elicitors tested here showed a dominant-enhancing property on cell growth in either variety. Moreover, high concentrations of CHI and LAM suppressed cell growth, especially in the PM cell suspension cultures.

In summary, the two plant varieties showed different growth responses and isoflavonoid accumulations after treatment with the elicitors. The differential response was especially prominent in the case of the oligosaccharide elicitor. The PM cell suspension was more sensitive than the PC cell suspension, and growth and isoflavonoid accumulation in the PM cells were more affected by elicitors than these processes in PC cells. To the best of our knowledge, this is the first time that the effects of abiotic and biotic elicitors on both *P. candollei* var. *mirifica* and *P. candollei* var. *candollei* have been reported. Our data on the effects of these elicitors in *P. candollei* cell suspension culture will be useful in enhancing the accumulation of isoflavonoid compounds in such cells and can also be used to further extend other plant culture models.

Acknowledgments This work was supported by The Thailand Research Fund (DBG4980009). SK is grateful for financial support from The Royal Golden Jubilee Ph.D. Program (PHD/0143/2548).

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