Enhanced isoflavones accumulation in methyl jasmonate-treated *in vitro* cultures of kudzu (*Pueraria lobata* Ohwi)

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Summary

Kudzu, *Pueraria lobata* (Willd.) Ohwi (Fabaceae) is a woody climber plant that naturally grows in tropical and subtropical East Asia, where the species is highly valued for its medicinal properties. Methyl jasmonate (MJ) stimulated isoflavones accumulation in *Pueraria lobata* whole plant cultures *in vitro* after 7 days of elicitation. Isoflavonoid content increased by the addition of 50, 100 and 200 μM of MJ. However, the plantlets growth was inhibited by increasing of MJ concentration. The elicitor (100 μM) enhanced total isoflavones production in plantlets of *P. lobata* to 10.44 mg/g dry wt. Moreover, MJ stimulated accumulation of daidzein and genistein. It was found that the total content of isoflavones increased in comparison with that found in the control plantlets, while content of genistein was 4–fold higher than control plants but the content of daidzein increased from undetectable level up to 0.54 mg.g^{-1} dry mass. The study shows that the content of active compounds in whole plant cultures of *P. lobata* may be enhanced by elicitation with MJ.

Key words: Pueraria lobata, *in vitro culture, elicitation, methyl jasmonate, isoflavonoids*

INTRODUCTION

Kudzu, *Pueraria lobata* (Willd.) Ohwi (Fabaceae) is a perennial, woody climber widely distributed in tropical and subtropical East Asia, where the species is highly valued for its medicinal properties [1]. The plant contains 21 different structures of pharmacologically active isoflavonoids distributed in whole plants: aglycones, O-glucosides and C-glycosides, mainly puerarin, daidzein, genistein, formononetin and daidzin [2, 3]. *Puerariae radix* is one of the most important oriental crude drugs in traditional Chinese and Japanese medicine which possesses multidirectional therapeutic properties and oestrogenic activities [3]. The isoflavones are the main active compounds of *Puerariae radix* (0.02–2%) along with tritrpenoidal sapogenins are responsible for its pharmacological properties. The crude drug increases coronary artery blood flow and is used as antipyretic, antidiarrhetic, diaphoretic, antiemetic, antispasmodic and antimicrobic remedy. Furthermore, it is active against angina pectoris, hypertension, deafness, optic nerve atrophy or retinitis and alcohol abuse agent [3]. *Puerariae Flos* is also used to counteract overconsumption of alcohol [4, 5]. Antioxidant and hypoglycemic activities of *P. lobata* crude extract were also reported [6]. The glucosides of isoflavones possess oestrogenic activities [7]. *Pueraria* root rich in phytoestrogens, is used as foodstuff in the Orient [8].

Isoflavones, common in the Fabaceae family, have many biological and pharmacological activities: anti-inflammatory, antimycotic and radical scavenging properties, and exhibit both estrogenic and antiestrogenic effects [9]. Genistein and daidzein and their derivatives inhibit the formation and growth of the breast and the ovaries tumours, which are directly related to the estrogenic balance within the body. These compounds can be used in cancer prevention and in postmenopausal treatment [9, 10]. Isoflavones have been classified as phytoalexins [11].

The method of *in vitro* multiplication of *Pueraria lobata* through axillary shoot proliferation from shoot tip explants followed by successful establishment of plants into the soil was described previously. Plantlets and callus tissues have showed the ability to produce isoflavones [12].

Accumulation of secondary plant metabolites which are unique sources for pharmaceuticals often occurs in plant subjected to stress including various elicitors or signal molecules [13].

Methyl jasmonate (MJ) is known as a signal compound produced after wounding or infection in plants. This compound has been shown to induce a variety of pathways leading to secondary metabolites which function as a defence compounds phytoalexins against pathogens [14]. Jasmonates can act as efficient elicitors of secondary metabolism in plant cell cultures. Elicitation was reported as a method to enhance the content of active compound in the tissue cultures of some medicinal plants. *In vitro* plant secondary metabolite production is enhanced through the application of a variety of elicitors to plant cell, tissue or organ culture system. However, no specific elicitor has a general effect on different plant species [13].

The aim of this study was to evaluate the effect of the elicitor – MJ on isoflavones accumulation in whole *in vitro* plant cultures of *P. lobata*.

MATERIALS AND METHODS

Plant material

The shoot tips from 4-week-old sterile plants growing on MS basal medium were used to initiate *in vitro* propagation of *Pueraria lobata*. (Willd.) Ohwi from seeds obtained from Tsukuba Plant Research Station, Japan. Multiplication of shoot cultures was performed on Murashige and Skoog (MS) [15] medium with 3% sucrose, 4.6 μM kinetin and 5.7 μM indole-3-acetic acid (IAA) as in previous paper [12]. The regenerated shoots were subcultured into 250 ml flasks containing 100 ml MS medium, without plant growth regulators for elongation and rooting. The cultures were maintained at 24 ºC under a 16-h light/day, at the intensity of 60 μmol·m^{-2}s^{-1} .

Elicitor treatment

For the experiments 23-day-old whole plant cultures of *P.lobata* were used. Elicitation was carried out with methyl jasmonate (MJ, Sigma) at various concentrations. MJ was dissolved in ethanol in order to prepare a stock solution. Separated rooted shoots (ca. 4 cm long) were transferred into 250 ml Erlenmeyer flasks with 100 ml liquid medium MS containing 4.6 μM kinetin and 5.7 μM IAA and MJ. Filter-sterilised MJ was added to achieve a final concentration of 50, 100 and 200 μM. Elicitor-untreated shoot cultures were used as control. The cultures were maintained on a gyratory shaker (100 rpm) at 24ºC under a 16-h light/day. Each of experiment was conducted twice.

Sample preparation and isoflavonoid analysis

Elicited and control plant materials were harvested after 7 days of culture and dried in the oven at 60ºC for thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analysis of isoflavones. Total isoflavones and individual compounds – genistein and daidzein content were determined by HPLC. The dry, powdered plant material (500 mg) was extracted three times with boiling methanol (MeOH, 15 ml). The MeOH extracts were evaporated *in vacuo*. The residues dissolved in water were partitioned between chloroform, butanol and water. The analysis for the presence of isoflavonoids was performed using Merck HP TLC silica gel plates Si60 F_{254}. Isoflavonoids were analyzed by TLC using $CHCl_3$- MeOH (19:1) and petroleum ether (40–50ºC) – diethyl ether (Et_2O) (1:1) as

solvent systems in comparison with authentic standards of daidzein, genistein, formononetin (Fluka) and puerarin (Sigma). Spots of compounds were analyzed under UV_{254} + NH_3 and after spraying with solution of $AlCl_3$, $FeCl_3$ or vanillin in H_2SO_4 , referring to literature data [16-18].

Quantitative analysis of isoflavonoids in hydrolyzed methanolic extracts was performed by HPLC method using a Merck-Hitachi D-7000 apparatus coupled with a photodiode array detector (DAD) and LiChrospher RP-18e column (250 x 4.6 mm; 5 μm (Merck). The isocratic solvent system was methanol and acidified water (ortho-phosphoric acid, pH=2.3). The flow rate was 0.8 ml/min and detection was performed at 248 nm. Retention times and UV spectra of determined isoflavones were compared to standards of genistein and daidzein. The result of analysis is the average value of three repetitions.

Statistical analysis

All results were means of three separate analyses from three samples of dried plant material for the estimation of isoflavonoids. Plant material derived from two independent experiments with six replicate flasks containing five plantlets in each treatment. The results were expressed as mg $.g^{-1}$ dry weight (DW).

Results (isoflavonoid content) were reported as the mean value s±95% confidence interval (standard error - SE x1.96 (quantile of normal distribution for probability of 95%) and the data were analysed by one-way analysis of variance (ANOVA) to compare means of group (0, 50, 100, 200 μM MJ) followed by mean separation using Tukey-Kramer Multiple Comparisons Test as a POST-HOC test. Differences with $p<0.05$ was consider as significant.

RESULTS AND DISCUSSION

Plant cell, tissue and organ cultures have been an attractive means for the production of bioactive compounds [19]. Altering various factors that influence a specific secondary metabolism in plant cultures can optimize the production of desired molecules.

Since production of secondary metabolites is, in general, higher in differentiated tissues, there is attempt to cultivate shoot cultures and whole plant cultures of *Pueraria lobata* for the accumulation of isoflavones. A previous paper describes procedure of *in vitro* propagation of *P. lobata* for successful elicitation of plantlets to increased isoflavones production [12].

The presence of three main isoflavones: daidzein, genistein and formononetin (fig. 1) characteristic for intact plants [20] has been confirmed in *in vitro* culture of micropropagated plantlets and callus of *P. lobata* by preliminary HPLC analysis of hydrolyzed methanolic extracts [12].

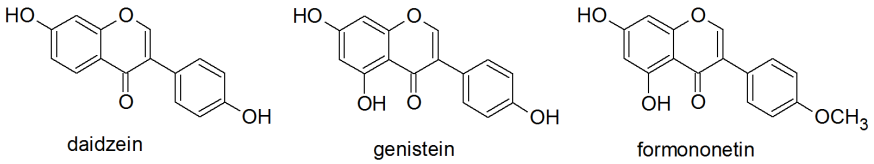


Figure 1. Chemical structures of main isoflavones present in *Pueraria lobata in vitro* cultures

Usually, plant cell cultures are widely used in order to activate secondary metabolites but there are few studies with plant organ culture system or whole plantlets regenerated *in vitro* [21-23]. The MJ was chosen because it is widely used as an elicitor in plant *in vitro* cultures [13]. Researchers have frequently used MJ at a concentration of 50, 100 and 200 μM to increase secondary metabolism in *in vitro* cultures [21, 23, 24].

In this study, *P. lobata* cultures were treated with various concentrations of methyl jasmonate. MJ elicitor was added to whole plant cultures to examine its effect on isoflavonoid accumulation (tab. 1). It improved isoflavonoids production after 7 days of treatment. MJ at 100 μM gave the highest concentration of isoflavones in studied plant material. The preliminary HPLC analysis of isoflavonoids revealed that contents of total and individual, non-conjugated isoflavones (genistein and daidzein) in MJ-treated whole plant cultures were increased compared to control. The HPLC chromatograms of extracts from whole plant cultures of *P. lobata* treated with MJ (50–200 μM) in comparison to control plantlets are shown in fig. 2. The treatment of cultures with 100 μM of MJ led not only to general increase of isoflavones but also to quantitative changes in isoflavone pattern. The MJ selectively enhanced the accumulation of genistein and daidzein in plant tissues. It was found that the isoflavonoids content increased. Although, differences between control values and the effects of total isoflavones were not statistically significant ($p=0.074$ is considered as non-significant). Compared to the control, the content of genistein in elicited plantlets increased significantly (4-fold) ($p=0.0011$). Variation among means calculated for 0 other values (50, 100, 200 uM MJ) is significantly greater than expected by chance.The content of daidzein increased from undetectable level up to 0.54 mg.g^{-1} dry mass. However, these results can only suggest the benefit effect of MJ because in this case ($p=0.7310$) differences between control values and content of daidzein in treated plantlets were not statistically significant.

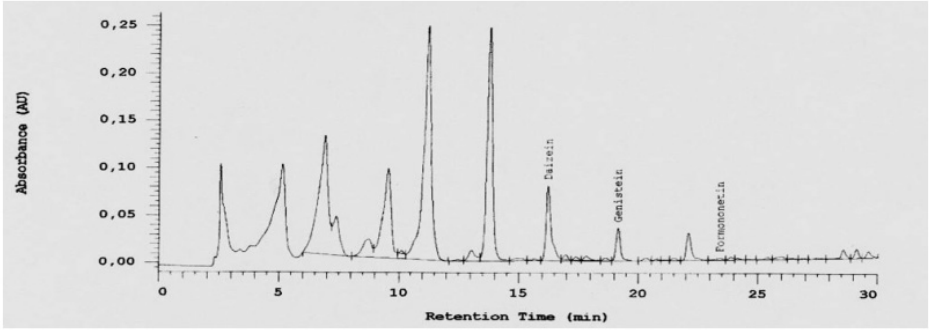
The highest isoflavonoid level was found in plantlets after 7 days of treatment with MJ (100 μM) which was almost twice the control. The effect of MJ on isoflavonoids production is shown in table 1. Daidzein was undetectable in studied control plantlets, whereas we found it in MJ elicited cultures (tab. 1). However, daidzein was found in the intact plants and *in vitro*-derived plants of *P. lobata*, in previous study [12]. Some differences in isoflavonoid content observed in plantlets and its reaction on MJ treatment can be explained by high genetic variance in that species [25]. Results of cytogenetic analysis of DNA content in *P. lobata* regenerants also showed some genetic changes of micropropagated plants [26].

Table 1.

Effect of methyl jasmonate concentration on isoflavonoid production in *P. lobata* after 7 days of elicitation. The results are expressed as mean values ±95% confidence interval

MJ concentration [μM]	average content ±1.96* SE [mg · g^{-1} DW]		
	total isoflavonoid compounds	genistein	daidzein
50	10.42±1.12	0.12±0.03	0.44±0.26
100	10.44±2.25	0.14±0.02	0.53±0.08
200	7.82±0.63	0.11±0.03	0.43±0.05
0 (control)	5.48±1.07	0.03±0.00	n.d.

* 1.96 – quantile of normal distribution for probability of 95%
SE – standard error
n.d. – not detected



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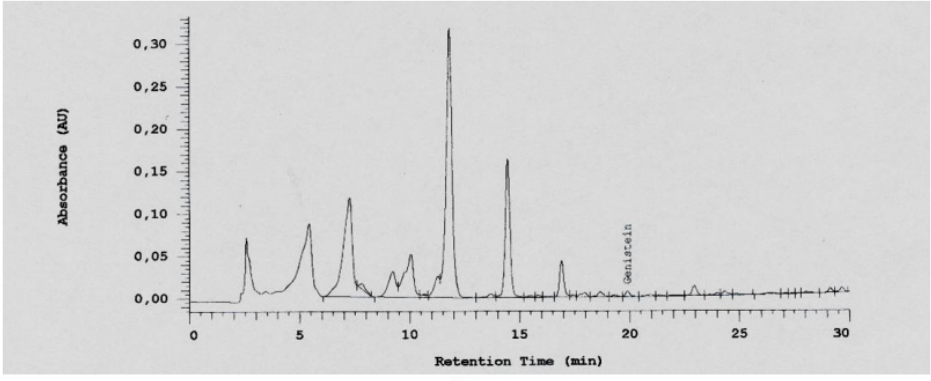

B

Figure 2. HPLC chromatograms of whole plant cultures of *P. lobata* A – culture after MJ treatment, B – control

Moreover, the effect of MJ on plant growth was measured as fresh and dry mass weight after treatment (30-day-old cultures). In all cases the growth capacity of treated plantlets declined in comparison to the control cultures (data not shown). These results are similar to those of Mangas et al. [19] who observed that plantlets treated with MJ showed a reduction of growth.

Isoflavonoids have been reported in cell, callus, hairy root cultures and propagated plants derived from a number of species from *Pueraria* genus [27-31]. In those studies, the efficacy of different factors was evaluated in order to optimize the production of isoflavonoids in *in vitro* cultures. Only a few reports on elicitation in *Pueraria lobata* plants have been published. The biosynthesis of isoflavonoids in elicitor–treated cell suspension, root cultures and stem of *P. lobata* have been studied [29, 30]. The authors showed that isoflavones accumulation was induced by variety of elicitors (yeast extract, $CuCl_2$, jasmonic acid, some biotic elicitors).

In our study, total isoflavonoid content of 10.44 $mg.g^{-1}$ dry mass and non-conjugated isoflavonoids - genistein content of 0.14 $mg.g^{-1}$ and daidzein of 0.54 $mg.g^{-1}$ obtained from organized cultures of *P. lobata* treated with MJ (100 μM) are in the same range to those recorded in optimized cell cultures of *Pueraria* species [27, 28].

Jasmonic acid (JA) and methyl jasmonate (MJ) are signal molecules that induce the plant's defense responses to pathogens or insects. They are produced and accumulated in plants, but exogenous application of JA and MJ can elicit secondary metabolite accumulation in defence response induction. JA and MJ are known as transducers of elicitor signal transduction that results in the biosynthesis of plant secondary metabolites. They act as inducers of phytoalexins [13, 14]. Accumulation of isoflavonoids, as phytoalexins, was enhanced in *in vitro* cultures of *P. lobata*. The accumulation of isoflavone phytoalexins has also been observed in *Phaseolus* species cultured *in vitro* [32].

In conclusion, these results suggest that MJ is an effective elicitor of isoflavones production in *P. lobata* cultured *in vitro*. Our findings indicate that the application of methyl jasmonate can exert a beneficial effect on isoflavonoids accumulation in *P. lobata in vitro* cultures.

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ZWIĘKSZANIE AKUMULACJI IZOFLAWONÓW W KULTURZE *IN VITRO* KUDZU (*PUERARIA LOBATA* OHWI) POD WPŁYWEM JASMONIANU METYLU

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S t r e s z c z e n i e

Kudzu *Pueraria lobata* (Willd.) Ohwi (Fabaceae) jest wartościową rośliną leczniczą, pnączem rosnącym dziko w tropikalnych i subtropikalnych rejonach Azji, gdzie gatunek jest wysoko ceniony z uwagi na właściwości lecznicze. Jasmonian metylu (MJ) zwiększał akumulację izoflawonów w kulturze *in vitro P. lobata* po 7 dniach elicytacji. Zawartość izoflawonoidów wzrosła po dodaniu MJ w stężeniu 50, 100 i 200 μM. Natomiast wzrost roślinek był hamowany wraz ze wzrostem stężenia elicytora. MJ (100 μM) zwiększył produkcję izoflawonów w zregenerowanych roślinkach *P. lobata* do 10,44 mg/g s.m Ponadto MJ stymulował produkcję daidzeiny i genisteiny. Stwierdzono, że całkowita zawartość izoflawonów wzrosła w porównaniu z ilością tych związków w roślinkach kontrolnych, podczas gdy zawartość genisteiny powiększyła się 4-krotnie, a daidzeina wzrosła z poziomu niewykrywalnego do 0.54 mg.g^{-1} suchej masy. Badania wskazują na możliwość zwiększania zawartości aktywnych związków w kulturze *in vitro* całych roślinek *P. lobata* w procesie elicytacji jasmonianem metylu.

Słowa kluczowe: *Pueraria lobata, elicytacja, jasmonian metylu, izoflawonoidy*