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Effect of methyl jasmonate on isoflavonoid accumulation and antioxidant enzymes in *Pueraria mirifica* cell suspension culture

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The effect of methyl jasmonate (MJ) on the isoflavonoid accumulation and activities of three antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) were studied in *Pueraria mirifica* cell suspension culture after MJ elicitation for up to 6 days. Treatment with MJ at a concentration of 1.0 µg/ml induced the highest isoflavonoid contents at the first day after elicitation, when compared to the control. This optimum concentration of MJ induced an oxidative stress in *P. mirifica* cells. The activity levels of superoxide dismutase and glutathione peroxidase were both increased, whilst that for catalase was decreased in the MJ-treated cells. Thus, *P. mirifica* cells have a defense mechanism against superoxide and hydrogen peroxide stress and MJ is one such potential stimulator of this defense mechanism.

Key words: *Pueraria mirifica*, isoflavonoid, methyl jasmonate, superoxide dismutase, catalase, glutathione peroxidase.

INTRODUCTION

The use of various herbs and medicinal plants in folklore medicine has a long history where they have been used since ancient times, especially in oriental countries (Sheng-Ji, 2001). However, the advent of antibiotics in the early 20th century led to a decline in their usage and a waned interest in providing scientific bases to their effects. The adverse effects of using antibiotics and other synthetic compounds on human health and on product quality and safety, plus increasing resistance to them, have regenerated interest in the fields of phytochemistry,

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phytopharmacology, phytomedicine and phytotherapy during the last decade (Makkar et al., 2009). Thus, more recently such folklore-based plants and their extracts are being evaluated for the chemical basis behind the treatment for adaptation to more conventional pure drug approaches or bioinformatic based modification, as well as for optimization of cultivar/cultivation conditions in agricultural rearing of the plants or biotechnological based mass production of the active component(s) (Chattopadhyay et al., 2004).

Plants produce numerous secondary metabolites that have historically been used as pharmaceuticals, fragrances, flavor compounds, dyes and agrochemicals (Sakuta and Komamine, 1988). An increasing number of these secondary plant metabolites are proving to be a major source of new drugs. Because their profiles vary among (even closely related) species, and also among cultivars and cultivation conditions, extracts from both known and newly discovered plants are subjected to screening for novel medical applications (Zhao et al., 2005). However, secondary metabolites are usually produced *in vivo* at only low concentrations, so large-scale production systems have been developed for use in plant cell culture.

Despite the enormous commercial efforts over several decades, only a few compounds have successfully been produced at a lower cost than by production by direct plant extraction or through chemical synthesis (Liu et al., 2002). Nevertheless, studies on secondary metabolite biosynthesis can increase the yield of active compound production in plants using biotechnology. Tissue culture techniques have been used to prepare primary cell cultures of plants in the form of either callus or cell suspension cultures, and from this, secondary metabolite production has been potentially developed via these cell culture techniques (Rao and Ravishankar, 2002).

P. mirifica, a Thai indigenous herb, with the local name of White Kwao Keur, has long been used among Thai women in a similar manner for modern hormone replacement therapy (Malaivijitnond, 2012). Products of White Kwao Keur are well known in international markets, such as China, Japan, Korea, Singapore, Malaysia, USA, Australia and Europe. Each year, the export value of "Kwao Keur" products from Thailand is approximately 1,500 million baht (~ 47.4 million US dollars). *P. mirifica* tuberous root contains a relatively rich diversity of isoflavonoids, including daidzein, daidzin, genestein, genistin and puerarin (Cherdshewasart et al., 2007). *P. mirifica* is classified as a medicinal plant but requires cultivation due to the limited sources of wild plants and habitat loss in their natural environment including soil erosion and deforestation (Sahavacharin, 1999). In addition, the amount of active components in the tuberous root has been reported to be dependent on various physical and chemical cultivation factors, such as the geographical location, climate and disease, as well as on the plant genetics (as in cultivar type) (Thanonkeo and Panichajakul, 2006). The variations in the level of active components, and their relative proportions, imposed by these factors have generated considerable interest in the use of the more controlled environment of plant tissue culture technologies for the production of isoflavonoids, so as to optimize and control these factors. These include the cultivation of specific organ cultures and suspension cultured cells selected for high production of secondary metabolites (Dicosmo and Misawa, 1995).

One way to increase the production level of secondary metabolites is by elicitation, which is the induction of secondary metabolite production by other molecules or treatments (the elicitors). Elicitors can be grouped into three categories. The first is the biotic elicitors, which are usually microbe-derived molecules that stimulate secondary metabolism. Biotic elicitors include polysaccharides, glycoproteins, low molecular weight organic compounds, and bacterial and fungal cell walls. Second are the abiotic elicitors, such as ultraviolet irradiation, salts of heavy metals and various chemicals. Thirdly are the endogenous elicitors, which are chemicals produced within the cell as secondary messengers, such as methyl jasmonate (MJ) (Fu et al., 1999). The optimal inclusion of elicitors in cell culture can lead to a pronounced increase in the activities of biosynthetic enzymes including differential effects on the enzymes involved in conjugate metabolism (Barz and Mackenbrock, 1994).

The effect of the elicitors depends on many factors, such as their concentration, the growth stage of the culture at the time of elicitation, period of contact and time course of elicitation. MJ has been found to enhance secondary metabolite production in plant cell cultures. This compound plays a key role both outside and inside plant cells. In exogenous application, MJ binds to the receptor on plant cell wall to stimulate biosynthesis of secondary metabolites, while intracellular MJ is one of the endogenous components in the jasmonic acid signaling pathway that is regarded as a transducer or mediator for elicitor signaling, leading to accumulation of plant secondary metabolites (Zhao et al., 2005).

The elicitation process also induced the reactive oxygen species (ROS) including superoxide (O_2^-) and hydrogen peroxide (H_2O_2). ROS may cause organelle, membrane and cell damage via oxidation of DNA, RNA, lipids and proteins. Plant cells have various antioxidant enzymes to restrict or reduce, and so act in defense of oxidative stress, such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GPx, EC 1.11.1.12). SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2, whilst CAT and GPx breakdown H_2O_2 into water and O_2 (Ali et al., 2006). Understanding the control of these enzyme activities in plant cells, as well as their biological and biochemical activities could also help explain and overcome

the enzymatic and non-enzymatic activities during cell cultivation and elicitation. The information would be useful for optimizing the efficient large-scale production of isoflavonoids in *P. mirifica* cell culture. Accordingly, it would be interesting to know the effect of elicitation with MJ upon the isoflavonoid accumulation and antioxidant enzyme activity of *P. mirifica* cell cultures. In this study, we investigated the role of MJ on isoflavonoid production and antioxidant enzymes in *P. mirifica* cell suspension culture.

MATERIALS AND METHODS

Plant material, cell culture and treatment procedure

P. mirifica seeds were obtained from Assoc. Prof. Yuthana Smitasiri (Guest lecturer, Graduate School, Chiang Mai University). The specimens were then authenticated by Mrs. Jantrararuk Towaranonte, a lecturer of the School of Science, Mae Fah Luang University and kept as voucher specimens No. MFLU-307. The seeds were sterilized by treating first with 95% (v/v) ethyl alcohol for 30 s, followed by 15% (w/w) Clorox® for 15 min and then rinsed three times with sterile distilled water. Seeds were germinated on Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose. Germinated seedling roots were placed on MS medium supplemented with 3% (w/v) sucrose, 0.5 µg/ml naphthylacetic acid (NAA) and 0.5 µg/ml benzyl adenine (BA) for callus induction. Cultures were maintained at 25 ± 2°C with a photoperiod of 16 h daylight at a photosynthetic proton flux intensity of 90 µmol m^{-2} s^{-1}. Cell suspension cultures were initiated by inoculation with 10 mg/ml of 4 weeks-old friable calli into a 125 ml Erlenmeyer flask containing 25 ml of liquid MS medium with 3% (w/v) sucrose, 0.5 µg/ml NAA and 0.5 µg/ml BA. The cultures were placed on a rotary shaker (125 rpm) under the same conditions as for the callus culture. The MJ solution was filter-sterilized before use and was aseptically added to the culture medium at one of three final concentrations (0.1, 0.5 or 1.0 µg/ml, equivalent to 0.45, 2.23 or 4.46 µM, respectively) during the 12th day of the cultivation. The control treatment contained sterile distilled water in the same quantities as the added MJ. Cells were collected everyday for 6 days after the addition of MJ (elicitation).

Growth measurement, extraction and analysis of isoflavonoids

The growth of cells was measured in terms of their dry weight (DW). The DW was measured after drying the fresh cells in an oven at 60°C for 3 days or until a constant weight was attained. The dried samples (10 mg) were ground and extracted with methanol (10 ml) for 24 h in room temperature, filtered through 0.45 µm filter paper and the methanol solvent removed from the extract in a hot air oven (60°C). The solid extract residue was then dissolved in methanol to a final volume of 1 ml before analysis. The isoflavonoid content was quantified by high performance liquid chromatography (HPLC) as described by Cherdshewasart et al. (2007). The system was operated on HPLC model SpectraSystem (Thermo Fisher Scientific, USA) equipped with autosampler model AS3000, Photodiode array UV detector model UV6000LP and Chromquest Software. The C18 column (250 mm × 4.6 mm, Luna 5U, Phenomenex, USA) was used with rational gradient eluding condition (86:14 to 68:32) (v/v) ratio of 0.1% (v/v) acetic acid: acetonitrile at a flow rate of 1 ml/min. Isoflavonoids were detected at 254 nm. For quantitative analysis, the system was calibrated with five authentic isoflavonoids (daidzein, daidzin, genistein, genistin and puerarin), purchased from Sigma-Aldrich Co. Standard curves were fitted with linear regression.

Extraction and assay for antioxidant enzyme activities

Fresh *P. mirifica* cells were ground in a mortar and pestle under liquid nitrogen and then extracted at 0 to 4°C in extraction buffer (50 mM phosphate buffer pH 7.0) at 0.2 mg/ml. The homogenates were clarified by centrifugation (10,000 × g, 4°C, 20 min), the supernatant harvested and used for enzymatic activity assays and determination of the protein content according to Bradford (1976), using bovine serum albumin as a standard. SOD activity was evaluated using the ribloflavin/nitrotetrazolium blue chloride (NBT) assay according to Lai et al. (2008). The inhibition of NBT reduction was determined by measuring the absorbance at 560 nm. One unit (U) of SOD activity was defined as the amount of enzyme that provides a 50% inhibition of the riboflavin-mediated reduction of NBT. CAT activity was measured at 240 nm by the H_2O_2 decomposition assay according to the method of Aebi (1974) in 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM H_2O_2 and 0.1 ml enzyme extract (at a total protein level of 0.5 mg/ml). GP_x activity was assayed by the oxidation of NADPH as described by Wendel (1980).

Statistical analysis

All parameters are shown as the mean ± standard error of the mean (SEM) of at least three independent experiments. The influence of various treatments on the plant cell biomass and isoflavonoid contents was processed statistically by analysis of variance (ANOVA) and the difference between means of the samples analyzed by Duncan's multiple range tests (DMRT) at a probability level of 0.05.

RESULTS AND DISCUSSION

The cell growth profile of *P. mirifica* cell suspension culture was established in preliminary trials, with the best cell growth being obtained in liquid MS medium supplemented with 0.5 µg/ml NAA and 0.5 µg/ml BA (data not shown). Under these conditions the biomass reached a maximum level at the 15th day (14.14 ± 0.17 g/L DW) of cell culture and decreased slightly thereafter, although the isoflavonoid production peaked after 9 days of culture (5.5 mg/g DW) before dropping at 12 days and then increasing from 18 days to a maximum level (6.0 mg/g DW) at 21 days of cell culture (Figure 1).

MJ treatment at 0.5 or 1.0 µg/ml increased the isoflavonoid accumulation with a greater response to the higher MJ concentration, in comparison with the control group. The maximum was 4 days after 0.5 µg/ml of MJ elicitation (5.2 mg/g DW and 1.24 times the control group), and it was only 1 day after 1.0 ug/ml of MJ elicittation (6.27 mg/g DW and 1.45 times of the control group) (Figure 2A). In contrast, at a concentration of 0.1 µg/ml MJ had no stimulatory effect upon isoflavonoid levels but

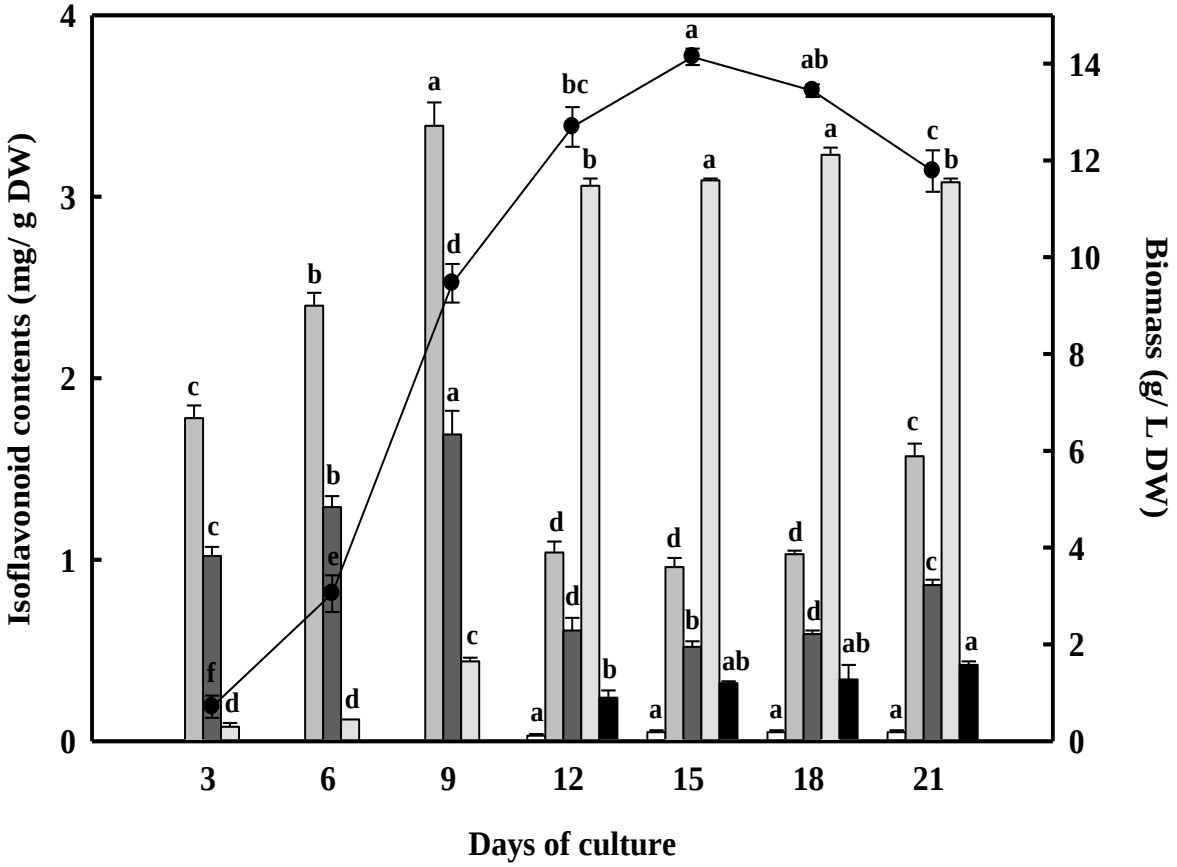

Figure 1. Cell growth (●) and isoflavonoid production: puerarin (white), daidzin (gray), genistin (dark gray), daidzein (light gray), and genistein (black) of *P. mirifica* cell suspension culture. Results are means ± SEM. Different letters in different bar differ significantly among samples (DMRT test, $P \leq 0.05$).

rather decreased the level at some time periods (especially at 6-day after MJ treatment). Knowledge of such periods of elicitation are useful for large scale production, such as in fed-batch or continuous bioreactors (Thanh et al., 2005; Ali et al., 2007; Prakash and Srivastava, 2008). This supports that MJ acts as a signaling molecule to induce the biosynthesis of isoflavonoid in *P. mirifica* cells, in agreement with the report of Korsangruang et al. (2010). However, the observed response on the total isoflavonoid levels is neither simply time nor dose-dependent. MJ treatment at all three tested doses did not affect the cell biomass of the *P. mirifica* cell culture (data not shown).

Bhagwath and Hjortso (2000) demonstrated that the production of thiarubrine A, a potential pharmaceutical, in the hairy root culture of *Ambrosia artemisiifolia* was enhanced eight-fold by elicitation with 50 µg/ml vanadyl sulphate, reaching 569 µg/g of biomass after 72 h. However, a higher maximum yield (647 µg/g of biomass) was achieved when the cultures were exposed to 5 mM autoclaved cell wall filtrates of *Protimyces gravidus*, a pathogenic fungus, for 48 h. The treatment of plant cells with biotic and abiotic factors is potentially a simple and effective means for stimulating secondary metabolite production in plant cell cultures. A variety of chemical and biochemical elicitors have been widely used to enhance isoflavonoid production in leguminous plant cultures. Kneer et al. (1999) reported that in the root cultures of *Lupinus leteus*, the genistin level was increased to 44.7 µg/g (8.94 times of the control group) when it was stimulated by 100 µM MJ. In addition, the production of genistin in the hairy root cell culture of *Psoralea* sp. were greatly enhanced with the addition of 30 µg/ml chitosan at the end of the exponential phase (Bourgaud et al., 1999).

MJ and its related derivatives have been implicated as signal transduction molecules with a multifaceted effect on plant growth, development and stress responses (Starwick, 1999). Moreover, the intracellular signal cascade that begins with the interaction of an elicitor molecule with the plant cell surface results, ultimately, in

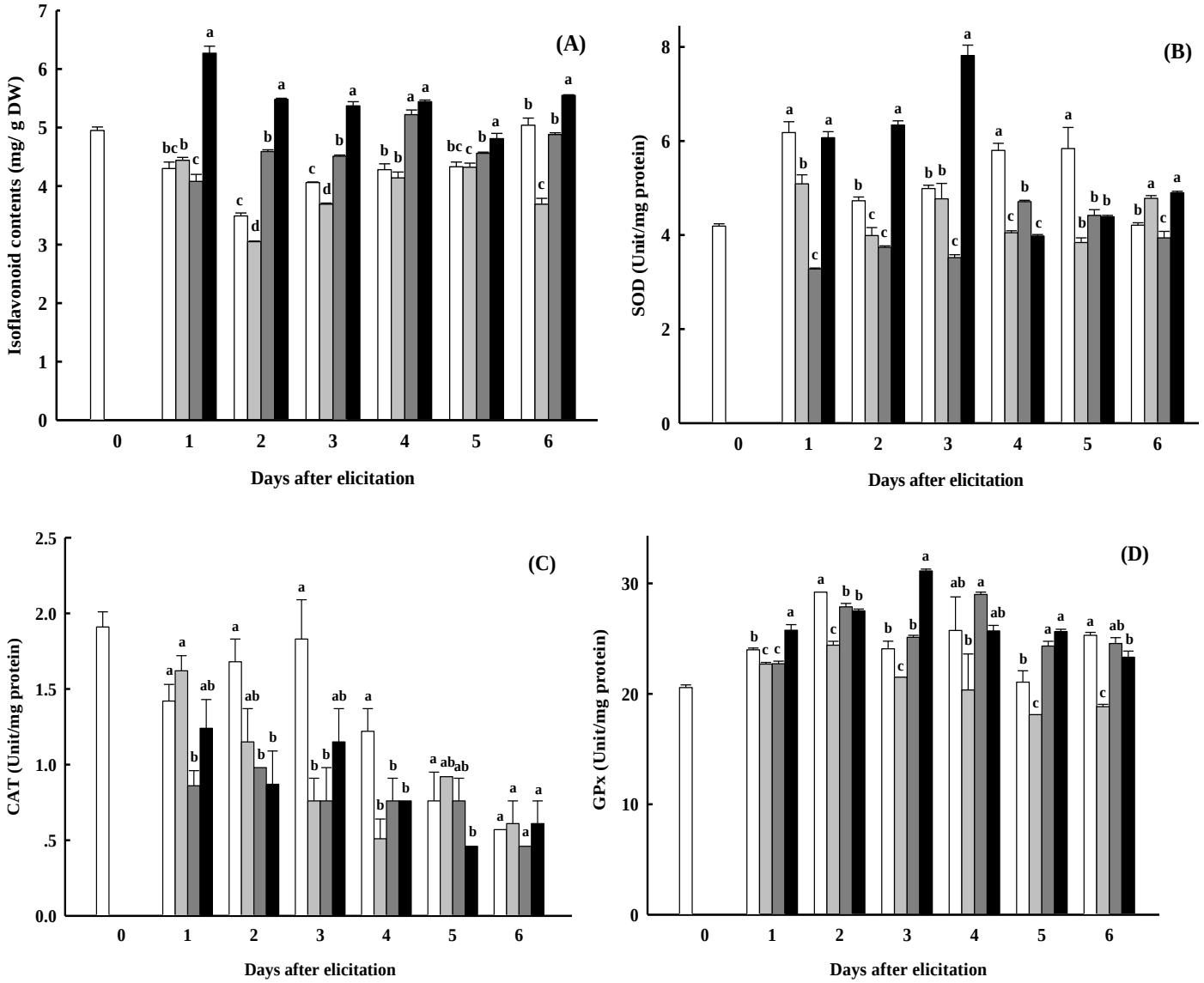

Figure 2. Effects of methyl jasmonate (MJ) addition at different concentrations on the (A) isoflavonoid production, and the enzymic activity levels of (B) superoxide dismutase (SOD), (C) catalase (CAT) and (D) glutathione peroxidase (GP_x); control (white), 0.1 µg/ml (gray), 0.5 µg/ml (dark gray), and 1 µg/ml (dark) in *P. mirifica* cell suspension culture.
Results are shown as the mean ± SEM. Different letters in different bar differ significantly among treatments (DMRT test, $P \leq 0.05$).

the accumulation of secondary metabolites (Gundlach et al., 1992). The role of MJ as an endogenous elicitor for stimulating secondary metabolite production in cell suspensions, and hairy root cultures of various secondary plants has been reported, such as for the lignans pinoresinol, metarisinol (Schmitt and Petersen, 2002), saponin (Lu et al., 2001) and ginsenoside (Yu et al., 2000), and alkaloids (Zabetakis et al., 1999). In addition, Furmanowa and Syklowaka-Baranek (2000) reported that paclitaxel was accumulated at high levels in hairy root cultures *of Taxus x media* var. *hicksii* after being elicited by 100 µM (22.43 µg/ml) MJ. Similarly, the production of terpenoid indole alkaloids in the hairy root cultures of *Catharanthus roseus* were increased by the same concentration of MJ (El-Sayed and Verpoorte, 2002).

The effect of MJ on the SOD, CAT and GP_x activities in the *P. mirifica* cell suspension culture are shown in Figure 2B to D. At a concentration of 1.0 µg/ml, MJ significantly

increased the SOD activity after two and three days exposure, reaching a maximum at the third day after elicitation (~ 7.8 U/mg protein), whereas in contrast, at a concentration of 0.1 or 0.5 µg/ml, MJ decreased the SOD activity compared to the control at all time points (Figure 2B). All MJ concentrations significantly decreased the CAT activity in the initial 1 to 4 days (or 1 to 5 days for 1.0 µg/ml) after elicitation (Figure 2C). The activity of GPx was significantly decreased by 0.1 and 0.5 µg/ml MJ, but was increased erratically over time after elicitation by 1.0 µg/ml MJ with a maximum level of GP_x activity on the third day after elicitation (Figure 2D).

Elicitation, especially at the highest dose of 1.0 µg/ml MJ causes oxidative stress via the production and accumulation of ROS. In this study, we focused on the enzyme activities of SOD, CAT and GPx since they are involved in the detoxification of ROS. MJ treatment at 1.0 µg/ml induced SOD and GPx activities by up to 56 and 30%, respectively, compared to the control treatment at the third day after elicitation. Whether the induction of SOD and GPx activity was correlated to an increase in the H_2O_2 and/or O_2^- levels, and so for the protection of the cell system (Jabs et al., 1997), is not investigated in this study. In contrast, the CAT activity was suppressed in MJ treatment while the GPx level was increased, which could suggest that the CAT functions in H_2O_2 removal were compensated by the GPx enzyme. A reduction in the level of CAT activity was also observed in *Panax ginseng* (Ali et al., 2006) and *C. roseus* (Zhao et al., 2001), with the activities of these enzymes reverting back to normal levels 6 days after elicitation.

Conclusion

This paper has described, for the first time, a procedure for initiation and establishment of cell suspension of *P. mirifica* which was able to accumulate a relatively high level of isoflavonoids and antioxidant enzyme activities. The treatment *P. mirifica* cell suspension culture have the highest isoflavonoid content and antioxidative enzyme levels on 1 and 3 days of culture with 1.0 µg/ml MJ, respectively. The results demonstrated the importance of the MJ (elicitor) concentration and time after addition in the growth medium. However, it seems that the increase of the isoflavonoid and antioxidative levels was not much in this study, the multiple elicitor additions over time or the addition of the multiple elicitors need to be tested. In addition, the cost of MJ compared to the value of the increased isoflavonoids obtained remains to be equated. Increasing the production level of isoflavonoid contents may be achieved by means of cultivation of *P. mirifica* in a large scale bioreactor, but this requires evaluation using the cell suspension cultures from our callus collection for confirmation and optimization of the production production of isoflavonoids under bioreactor conditions.

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ABBREVIATIONS

BA, Benzyl adenine; **CAT,** catalase; **GPx,** glutathione peroxidase; **MJ,** methyl jasmonate; **MS,** Murashige and Skoog's medium; **NAA,** naphthylacetic acid; **NADPH,** nicotinamide adenine dinucleotide phosphate; **NBT,** nitrotetrazolium blue chloride; **ROS,** reactive oxygen species; **SEM,** standard error of the mean; **SOD,** superoxide dismutase.

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