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Stimulatory effect of methyl jasmonate and squalestatin on phenolic metabolism through induction of LOX activity in cell suspension culture of yew

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Abstract: Cell suspension cultures of *Taxus baccata* were treated with 2 elicitor compounds, methyl jasmonate (MeJA) and squalestatin (S), individually and in combination for 7 days to determine if they mediated the enhancement of biosynthesis of endogenous jasmonate through induction of lipoxygenase (LOX) activity. Total phenolic compounds, total flavonoids, total antioxidants, phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and LOX activities in 5-month-old yew cell cultures were studied. Our results showed that MeJA and S could stimulate production of phenol derivatives in cell suspension cultures of *T. baccata*. In parallel to the induction of phenolic production in elicited cells, results showed that activities of PAL and PPO enzymes and total antioxidants significantly increased in *Taxus* cells in response to MeJA and S. Maximal activities of lipoxygenase were obtained 48 h after treatment with MeJA (100 µM), S (0.1 µM), and the combination of the 2 elicitors. Results showed that MeJA and S are effective elicitors for increasing phenolic production in *Taxus* cell suspension cultures, likely through increasing LOX activity followed by an increase in endogenous jasmonate.

Key words: Antioxidant, lipoxygenase, phenylalanine ammonia-lyase, squalestatin, *Taxus*

1. Introduction

Biotic elicitors are commonly used to stimulate the processes leading to production of secondary metabolites in cell culture systems. In particular, jasmonic acid or its methyl ester (MeJA) have been effective in enhancing alkaloids, terpenoids, and phenolics in *Nicotiana* spp., *Hyoscyamus muticus*, and *Taxus baccata* (Yukimune et al., 1996; Moon et al., 1998; Singh et al., 1998; Keinänen et al., 2001; Bonfill et al., 2006). Squalestatin (S; zaragozic acid) is a fungal antibiotic and an inhibitor of squalene synthase enzyme. It was isolated from terrestrial fungi during identification of novel inhibitors of cholesterol biosynthesis from natural sources (Procopiou et al., 1994). Application of elicitors derived from microorganisms has been considered to be one of the most effective strategies for improving the production of useful secondary metabolites in plant cell cultures.

Plant response to stressful environmental conditions and avirulent pathogens may be evaluated by activation of defense-related genes, deposition of lignin, and synthesis of specific compounds such as phenolics that confer higher detoxification of reactive oxygen species in the plant cells (Mittler, 2002).

As an intermediate signal, jasmonic acid has been implicated in elicitor-induced secondary metabolite accumulation in plants (Gundlach et al., 1992; Nojiri et al., 1996). Linolenic acid is utilized by plant cells as a substrate for the lipoxygenase (LOX)-dependent synthesis of jasmonic acid (Vick and Zimmerman, 1984). Hydroperoxidation of specific unsaturated fatty acids is catalyzed by LOX. There have been several reports of LOX induction by MeJA (Grimes et al., 1992; Melan et al., 1993). Induction of LOX by jasmonate may be important for increasing jasmonate levels under certain growth conditions. Jasmonic acid accumulation is one of the common responses of plant cells to fungal elicitors (Creelman, 1997).

Synthesis of phenolic compounds is recognized as a result of signaling processes initiated very quickly after injury, attack of pathogens, or elicitation. The initial step of phenylpropanoid synthesis is mediated by phenylalanine ammonia-lyase (PAL) enzyme. An increase in PAL activity could often be considered as a marker of plant reaction to environmental stress. Jasmonate induces genes involved in phytoalexin biosynthesis (*Pal*) (Creelman et al., 1992) and phenolics (polyphenol oxidase) (Doares et al., 1995) that

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are involved in plant defense. Polyphenol oxidase (PPO) is widely distributed throughout the plant and plays a role in oxygen scavenging and defense against stress (Esen et al., 1993). Phenolic compounds and especially flavonoids constitute the main components of the antioxidant system in plants (Zheng and Wang, 2001). The antioxidant activities of phenolic compounds are mainly due to their redox properties.

In this study, we selected elicited cell suspension cultures of *T. baccata* to gain new insight into the regulatory role of LOX in the metabolism of phenolic compounds exposed to MeJA and S.

2. Materials and methods

2.1. Cell culture

Taxus baccata cell suspension was initiated from adult leaves as previously described by Kajani et al. (2010). Briefly, the leaves were cultured on Gamborg's B5 medium containing 2 mg/L naphthalene acetic acid, 0.2 mg/L 2,4-dichlorophenoxyacetic acid, 0.2 mg/L kinetin, 500 mg/L polyvinylpyrrolidone (PVP), 30 g/L sucrose, and 10 g/L agar and were kept for 5 months at 25 °C in darkness for callus induction. Homogeneous calli were obtained from several subcultures in the same medium. To determine the effects of MeJA (Sigma-Aldrich, Inc.) and S (Sigma-Aldrich, Inc.) on phenolic production, 1 g (fresh weight) of calli was inoculated into a 250-mL flask containing 100 mL of B5 medium. All the flasks were aerated in the same shaker at 110 rpm in the dark. MeJA was dissolved in EtOH and prepared as a concentrated stock solution; 100 µM MeJA was added to the 21-day-old cell suspension cultures. S was dissolved in 50 mM potassium phosphate (pH 7.5) and prepared as a concentrated stock solution. Two concentrations of S (0.1 µM and 1 µM) were added to 21-day-old cell suspension cultures. A combination of MeJA (100 µM) and squalestatin (1 µM) was the fourth treatment, which was applied to 21-day-old cell suspension cultures. Each treatment was supplied with separate controls at identical concentrations to the treatment.

2.2. Biomass accumulation

One week after elicitation, the cells from a sampled shake flask were washed with a large amount of distilled water. Cell biomass was then harvested by sieving the suspension cultures through 40-µm mesh to measure fresh weights.

2.3. Total phenolic compound extraction from the cells and medium

A ground freeze-dried sample of 0.1 g was weighed, and phenolic and flavonoid compounds were extracted with 50 mL of 80% aqueous methanol on a shaker for 24 h at room temperature. The extracts were centrifuged for 15 min at 14,000 rpm. In order to extract from the culture medium, each culture medium was mixed with an equal volume of methanol (80%) and was placed on the shaker for 24 h at room temperature. The resulting mixture was concentrated in the rotary at 50 °C. The concentrated extract was mixed with 2 mL of methanol and was passed through a 0.22-µm filter.

The total phenolic content of the extracts was determined by using the Folin–Ciocalteu assay (Singleton and Rossi, 1965). First, 300 µL of extract was placed in a reaction test tube, to which 1.5 mL of Folin–Ciocalteu reagent was added. The test tube was allowed to stand for 5 min, and then 1.2 mL of 7.5% Na_2CO_3 was added. After 30 min in the dark, absorbance was measured at 750 nm. Total phenolic content was expressed as g gallic acid equivalents/100 g dry weight (DW).

Total flavonoid content was measured by the aluminum chloride colorimetric assay (Zhishen et al., 1999). An aliquot (1 mL) of extract or the standard solution of catechin was added to a test tube containing 4 mL of water; 300 µL of 50% $NaNO_2$ was then added to the test tube. After 5 min, 300 µL of 10% $AlCl_3$ was added. After 6 min, 2 mL of 1 M NaOH was added and the total volume was made up to 10 mL with H_2O. The solution was mixed well, and the absorbance was measured against prepared reagent blank at 510 nm. Quantification was done with respect to the standard curve of catechin. The results were expressed as g catechin equivalents/100 g DW.

2.4. Preparations and assays of enzymes

One hundred milligrams of fresh cells was homogenized in 1.5 mL of 50 mM sodium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. After centrifuging at 12,000 × *g* for 20 min at 4 °C, the supernatant was used as a source for PAL and PPO activity assessments.

2.4.1. PAL activity

PAL function was assayed according to Zucker (1965). The reaction mixture contained 1 mL of 0.05 M Tris-HCl buffer (pH 8.0), 0.1 mL of enzyme extract, 0.5 mL of 10 mM L-phenylalanine, and water to a total volume of 3 mL. After 1 h of incubation at 37 °C, the reaction was stopped by the addition of 0.1 mL of 1 N HCl. Increase in absorbance due to PAL activity was recorded spectrophotometrically at 290 nm. PAL activity was expressed as units/g fresh weight.

2.4.2. PPO activity

The extract (50 µL) was mixed in a quartz cuvette with 1.95 mL of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 M catechol as a substrate. PPO activity was assayed spectrophotometrically based on an initial rate of increase in absorbance at 410 nm (Soliva et al., 2001).

2.4.3. LOX activity

LOX activity was assayed according to Axelrod et al. (1981). One hundred milligrams of the fresh cells was homogenized in 1.5 mL of 0.1 M Tris-HCl buffer (pH 8.5)

containing 1% PVP (w/v), 1 mM $CaCl_2$, and 10% (v/v) glycerol. After centrifuging at 11,000 × *g* for 20 min at 4 °C, the supernatant was used as a source for the crude enzyme activity test. LOX activity was measured in a reaction mixture consisting of 1.95 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 50 mM linoleate and 50 µL of enzyme extract. The change of absorbance at 234 nm was measured per minute at 25 °C, and the results reported were based on ΔOD g fresh weight^{-1} min^{-1}.

2.5. Total antioxidant activity

Methanolic extract sample (0.3 mL) was mixed with 3.0 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The reaction mixture was incubated at 95 °C for 90 min in a water bath. Absorbance of all the sample mixtures was measured at 695 nm. Total antioxidant activity is expressed as g ascorbic acid equivalents/100 g DW (Ganesan et al., 2008).

2.6. Statistical analysis

Experiments were replicated 3 times, and statistical significance was measured by using the one-way analysis of variance test (ANOVA). Least significant different (LSD) mean comparison was used to test significant differences between treatments at the 5% level. All statistical analyses were performed with SAS software (SAS Institute Inc., Cary, NC, USA, 1999).

3. Results

3.1. Effect of MeJA and S on fresh weight, total phenolics, flavonoids, and antioxidant contents

The growth index of the cell suspension cultures of *T. baccata* was determined by using the measures of the dry weights 7 days after the addition of the elicitors. Results showed that there were no significant ($P < 0.05$) differences in the growth of cells under different treatments (data not shown).

Measurement of total phenolic compounds (intracellular + extracellular) showed that there were significant differences ($P < 0.05$) among the treatments (Figures 1A–1C). The maximal levels of phenolic compounds were obtained in both MeJA + S and S 0.1 treatments, which produced 3.56 g/100 g DW and 3.51 g/100 g DW, respectively, 2.94 and 2.90 times higher than in controls. Cells treated with MeJA also produced 3.09 g/100 g DW of phenolic compounds. However, no significant ($P < 0.05$) changes were observed in total phenolic compounds of cells treated with S 1.

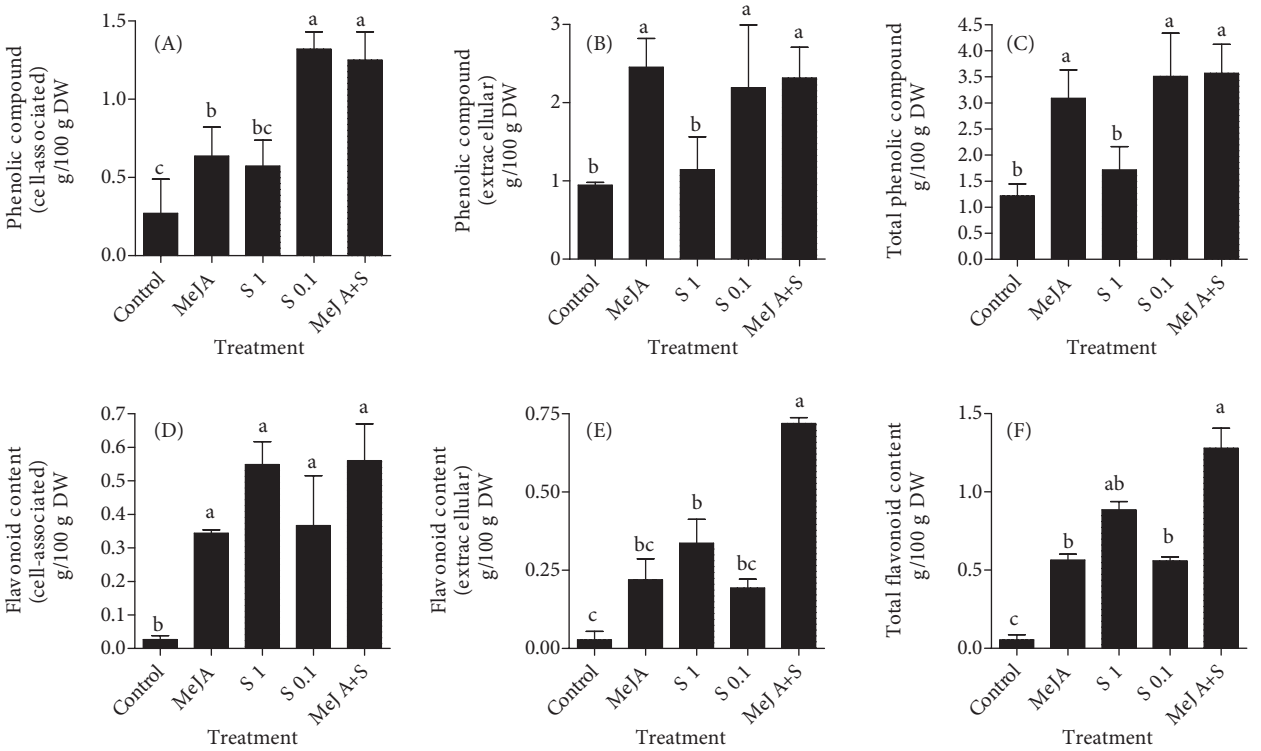

Figure 1. Effect of MeJA and S on total (cell-associated + extracellular) phenolic (g/100 g DW) (A, B, and C), and total (cell-associated + extracellular) flavonoids (g/100 g DW) (D, E, and F) in cell suspension culture of *Taxus baccata* after 1 week. MeJA: 100 µM methyl jasmonate; S 1: 1 µM squalestatin; S 0.1: 0.1 µM squalestatin; MeJA+S: 100 µM methyl jasmonate + 1 µM squalestatin. Values are means of 3 replicates. Common letters over bars indicate that data are not significantly different ($P < 0.05$) by LSD test.

The pattern of changes in extracellular phenolic compounds was similar to that of the total phenolic compounds. Treatment S 1 did not show a significant difference compared to its control, and other treatments significantly increased the extracellular phenolic compounds (Figure 1). Results showed that MeJA treatment increased the extracellular phenolic compounds to up to 79.39% of the total phenolic compounds, which was the highest among the applied treatments. This treatment could be considered to be the most capable of excreting more phenolic compounds into the medium. The contents of intracellular phenolic compounds in the *T. baccata* cell cultures were also determined individually (Figure 1). Intracellular phenolic compounds were only increased for treatments S 0.1 and MeJA + S (1.32 and 1.25 g/100 g DW, respectively); they did not show significant changes with MeJA and S 1 treatments (0.63 and 0.57 g/100 g DW) compared to controls.

Results showed that all of the treatments significantly increased the total (intracellular + extracellular) flavonoids produced (Figures 1D–1F). The maximum amount of total flavonoid was measured for the MeJA + S treatment, which produced 1.27 g/100 g DW, 23.6 times higher than its control. Cells treated with S 1, MeJA, and S 0.1 produced 0.885, 0.563, and 0.559 g/100 g DW of phenolic compounds, respectively. The only treatment that resulted in a significant change (increase) in extracellular flavonoid was MeJA + S. Cells under this treatment produced 0.718 g /100 g DW, which equals 56.2% of the total flavonoid production in this treatment. Other treatments, S 1, MeJA, and S 0.1, produced 0.336, 0.219, and 0.193 g/100 g DW of extracellular flavonoids, respectively, none of which were significantly different from controls. All the treatments significantly and equally increased the intracellular flavonoids compared to controls (Figure 1). The amounts of intercellular flavonoid for S 0.1 and S 1 treatments were 65.46% and 61.9% of the respective total flavonoid and were 60.9% and 43.8% of respective total flavonoid for MeJA and MeJA + S treatments.

Antioxidant activities of elicited cell extracts in methanol are shown in Figure 2. Elicitation of the *T. baccata* cell cultures with MeJA and S exhibited significant induction of antioxidant activity. Total antioxidant activity in the MeJA + S treatment was about 4-fold greater than that of the control. The correlation between total antioxidant activity and total phenolic contents of elicited cells had a correlation coefficient of $R^2 = 0.92$. A high correlation between antioxidant capacities and their total phenolic contents indicated that phenolic compounds were a major contributor to the antioxidant activity of *Taxus* cells.

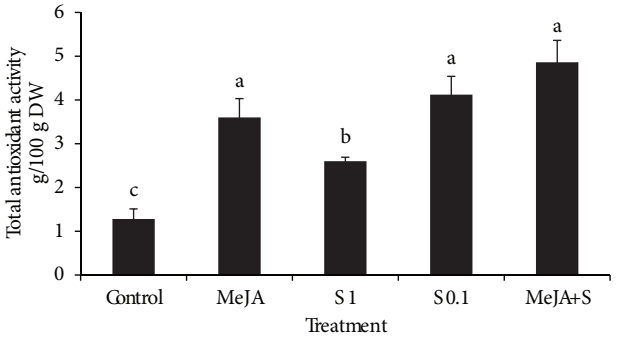

Figure 2. Effect of MeJA and S on total antioxidant activity (g ascorbic acid/100 g DW) in cell suspension culture of *Taxus baccata* after 1 week. Other details as in Figure 1.

3.2. Effects of MeJA and S on PAL, PPO, and LOX enzyme activities

Our results showed that all the treatments significantly increased the activity of PAL (Figure 3). The highest activity was measured for the MeJA + S treatment, which was 1.63 times higher than in the control. PAL activity in cells treated with S 0.1, MeJA, and S 1 was 1.37, 1.46, and 1.24 times higher than in the respective controls.

The effects of MeJA and S on PPO activity in *T. baccata* suspension culture are shown in Figure 4. A significant (P

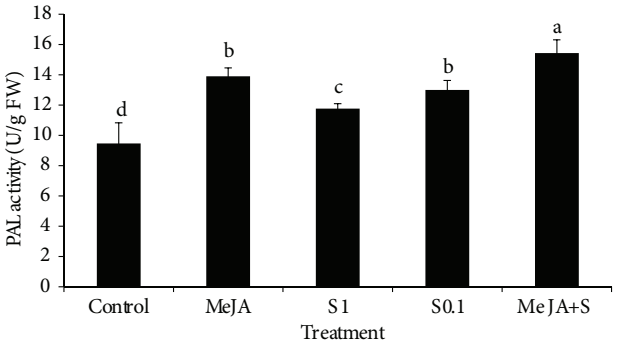

Figure 3. PAL activity in cell suspension culture of *Taxus baccata* treated with MeJA and S. Other details as in Figure 1.

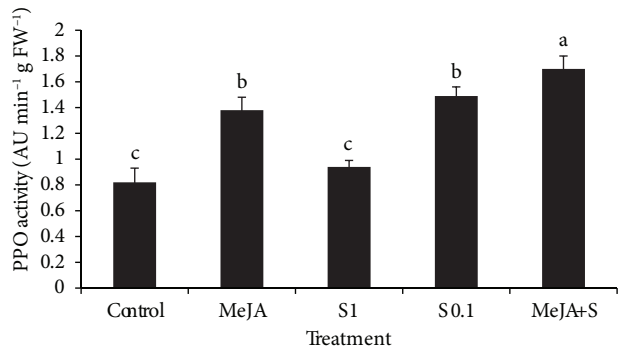

Figure 4. PPO activity in cell suspension culture of *Taxus baccata* treated with MeJA and S. Other details as in Figure 1.

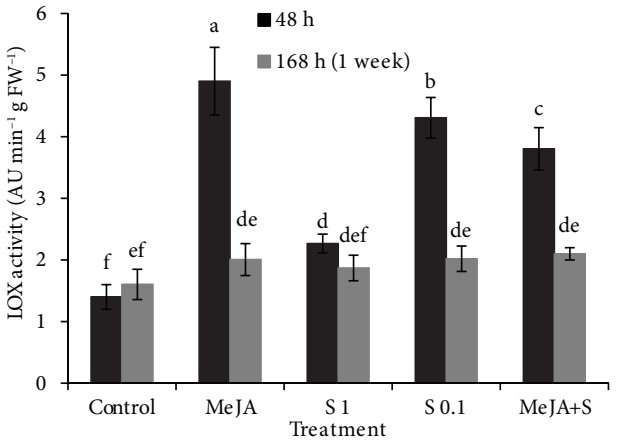

Figure 5. Effect of MeJA and S on LOX activity in cell suspension culture of *Taxus baccata* after 2 and 7 days. Other details as in Figure 1.

< 0.05) increase in PPO activity was observed after 7 days of exposure to MeJA, S, and their combination.

A marked increase in LOX enzyme activity was observed after 46 h in cells elicited with MeJA, S, or their combination. Activity of the enzyme in all treatments decreased to control levels after 168 h (Figure 5).

4. Discussion

Production of taxane in cell suspension cultures of *Taxus* spp. has been extensively studied; however, other secondary metabolites of this plant have not been sufficiently studied. Major phenolic compounds of *T. cuspidata* were summarized in a review work by Wang et al. (2010). Bulgakov et al. (2011) reported up to a 2-fold increase in catechin and epicatechin flavonoids in cell suspension cultures of *T. cuspidata* under elicitation with MeJA. In the current study, increased phenolic and flavonoid compounds were observed in cell suspension cultures of *T. baccata* under MeJA and S elicitations, with the greatest increase observed for the combination of the 2 elicitors (MeJA + S). These observations were concordant with previous reports by Kim (2007) and Ming (2009), who reported increased phenolic compounds under MeJA treatments, or those by Yuan et al. (2002), Shouwei et al. (2009), and Koç and Üstün (2012), who reported the same for fungal elicitation; all of these authors considered the increased production of phenolic compounds to be part of the plant defense response system.

In general, phenolic compounds reflect the antioxidant activity in stressed plants and may contribute to protecting cells from the oxidative damage of free radicals by reducing their toxicity on cytoplasmic structures (Mittler, 2002; Wahid and Ghazanfar, 2006). The correlation between antioxidant capacity and the total phenolic content in medicinal plants has already been reported by Li et al. (2008). Positive linear correlation ($R^2 = 0.92$) was observed between phenolic compound constituents and antioxidant capacity in cells elicited with both elicitors.

In parallel to the induction of phenolic production in elicited cells, the results in this study showed that the activity of PPO enzyme significantly increased in *Taxus* cells in response to MeJA and S. The highest activities of PPO enzyme were measured for the cells elicited with the combination of both MeJA and S.

In this study, treatment of *T. baccata* cell cultures with MeJA and S increased phenolic compounds and flavonoids. It is well known that these compounds are produced in the phenylpropanoid pathway, with PAL as the key enzyme of the pathway. Treatment of cell suspensions of *T. chinensis* by the fungal elicitor extracted from *Fusarium* led to induction of the phenylpropanoid pathway and increased production of PAL 36 h after elicitation (Yuan et al., 2002).

In addition, the enhancement of PAL activity in plant cell culture treated with MeJA has been proven. For example, Walter et al. (2002) reported that the activation rate of enzymes attributed to plant defense, such as PAL and peroxidase, increased in *Hordeum* leaves after treatment with MeJA. Conceição et al. (2006) also showed that the enhancement of phenolic compound synthesis in cell culture of *Hypericum perforatum* can be due to MeJA. In comparison to control cultures, MeJA and S could stimulate production of phenol derivatives in cell suspension cultures of *T. baccata*. It was observed that the rise in PAL activity correlated with accumulating levels of total phenol compounds in cultures.

The role of S as a fungal elicitor for plant secondary metabolites is not clear. However, the involvement of the octadecanoid pathway in fungal and elicitor-inducible production of secondary metabolite was shown in *Catharanthus* (Menke et al., 1999) and *Silybum* (Khalili et al., 2009). Gundlach et al. (1992) and Blechert et al. (1995) also reported that endogenous jasmonate was accumulated after fungal elicitor treatment of cell cultures. LOX enzyme activity was evaluated in elicited cells of *T. baccata* to determine if S and MeJA had played their roles via enhancement of the biosynthesis of endogenous jasmonate. Results showed that enzyme activity 48 h after treatment was increased for both elicitors, which indicated that MeJA and S triggered jasmonate biosynthesis in *T. baccata* cells. Grimes et al. (1992) reported that lipoxygenase enzyme activity was increased (25%) in soybean in vitro cultures under MeJA treatment. Induction of LOX activity in leaves of tobacco treated with cryptogein (a protein extracted from *Phytophthora cryptogea* fungi) was similarly reported by Rusterucci et al. (1999).

The desired effects of S and MeJA might be due to changes in LOX enzyme activity in elicited cells of *T. baccata*. In other words, increased LOX activities in elicited

cells caused by MeJA and S treatments may be connected to increased endogenous jasmonate, which improved the defense system (by phenolic compound production) in elicited cells.

It was concluded that MeJA and S elicitors increased the production of phenolic compounds in cell suspension cultures of *T. baccata* through activation of the octadecanoid biosynthetic pathway. Application of combined elicitors (MeJA + S) resulted in additive effects of each elicitor.

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