The Stimulation of the Anticancer Chemical Taxol in *Taxus baccata*

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Abstract

Taxol is an anticancer drug that is widely used in cancer treatment worldwide. Since the source of the drug is a plant, the increase in its production is one of the concerns of researchers. One way to increase the production of Taxol is to use stimulants for secondary metabolite production. Therefore, in this study, methyl jasmonate was used as a bioelicitor, and its effect on Taxol production was evaluated. For this purpose, *Taxus baccata* was elicited for 48 and 72 hours with concentrations at 0, 100, 250, and 500 μM methyl jasmonate, and the quantities of Taxol in the leaf and stem explants were measured by high-performance liquid chromatography (HPLC). The results showed that among the leaf and stem explants, a tremendous amount of Taxol production is related to the leaf, which is a great advantage in reducing the number of trees cut down to obtain Taxol. In addition, the 48-hour elicitation time demonstrated the best result in the production of Taxol. The optimal concentration of methyl jasmonate was also estimated at 500 μM and 0.326 mg/g of dried leaf biomass. The results of this experiment showed that methyl jasmonate has the potential to be used as a biological elicitor to produce considerable amounts of Taxol in *Taxus baccata*.

Forests are rich in resources such as wood, herbal medicines, natural dyes, food products, and other things humans have used for years. Traditional herbal medicines date back thousands of years. Chinese herbal medicines have not only had various therapeutic uses, but in many cases have also helped maintain the environment's health (Zhu et al. 2021). Medicinal plants are of particular importance; they supply more than 25 percent of all drugs used worldwide, including anticancer drugs (Haghiroalsadat et al. 2011). Drugs such as Taxol, Vinblastine, Vincristine, Camptothecin, and Podophyllotoxin are among the most important anticancer drugs of plant origin. Taxol and its related compounds (Taxanes) or (Taxoids) are crucial for treating all types of cancer, including ovarian, breast, lung, and AIDS-related Kaposi sarcoma (Lee et al. 2011, Lichota and Gwozdzinski 2018). Moreover, numerous studies and clinical trials have demonstrated the effectiveness of these medicinal substances on other diseases, including polycystic liver disease. The discovery of the therapeutic effects of these compounds against many diseases could generate a market demand of billions of dollars (Cicenas et al. 2015).

The natural source of Taxanes is species of the genus *Taxus* (*Taxus* sp.). The yew (*Taxus baccata*) is a conifer tree of the Taxaceae family, one of the native species in the northern forests of Iran (Ahmadi et al. 2020). Every year, millions of stems of this tree are commercially cultivated in France and America by four major pharmaceutical companies, Bristol-Myers Squibb (https://www.bms.com/), Mylan (www.mylan.com), Phyton Biotech (https://phytonbiotech.com), and Sanofi-Aventis (https://www.sanofi.com/), to extract the anticancer substance Taxol. However, because of its rarity, slow growth, and shallow Taxol content in natural plants, as well as the presence of over 400 similar compounds that make the purification process very complex, extraction of these substances from the plant is not a cost-effective method, and it is being replaced by other methods. Chemical synthesis, semisynthesis, and microbial production are among the methods which have generally replaced earlier methods. However, production efficiency is very low due to the production of many unwanted isomers, large fluctuations in the production rate of precursors, and

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relatively small production in relation to the plant's size (Cravens et al. 2019, Ke et al. 2021).

Taxanes are among the secondary metabolites and diterpene compounds that protect the plant from injury and pathogenic agents. The accumulation of secondary metabolites in plants is a defensive response to pathogen attacks induced and activated by stimulants. Stimulants commonly used in the studies include fungal carbohydrates, yeast extract, methyl jasmonate (MeJA), salicylic acid, and chitosan (Ramirez-Estrada et al. 2016). Jasmonates, including jasmonic acid and MeJA, form a group of cyclopentanone compounds that modulate a wide range of plant responses and act as effective inducers for improving secondary metabolite production in laboratory cultures (Creelman and Mullet 1997, Sembdner and Parthier 2003). They represent an essential class of elicitors for numerous secondary metabolic pathways, typically demonstrated by inducing the biosynthesis of secondary metabolites when plants are exposed to specific environmental stresses (Pauwels et al. 2009).

Signaling molecules such as salicylic acid (SA), jasmonic acid (JA), and MeJA are internal plant growth regulators which play a crucial role in plant growth and response to environmental stresses. These signaling molecules, when used externally, also move along the plant in some way and trigger the expression of specific defense genes in the plant (Aslam et al. 2021; Balestrini et al. 2021). Twenty years after the first recognition of jasmonic acids, their first physiological effect was identified. These substances were recognized as senescence promoters, growth inhibitors, and stimulators of secondary metabolism in various plant species (Chandran et al. 2020). Jasmonic acid and its derivatives induce the activity of specific enzymes catalyzing biosynthetic reactions related to the production of defense compounds such as polyphenols, alkaloids, and pathogenic bacterial proteins (Andi et al. 2001).

Little information is available on controlling the Taxol pathway at the transcriptional, translational, and posttranslational levels. Elicitors such as methyl jasmonate have long been used to improve secondary metabolism in Taxus cell cultures. However, the mechanisms by which MeJA activates Taxol biosynthesis have not been elucidated in detail. Knowledge of regulatory mechanisms, identification of key transcription factors, and any feedback loops in the pathway are critical in informing metabolic engineering efforts (Mutanda et al. 2021). This richness of information will be crucial in designing the biosynthetic pathway to Taxol, particularly in plant cells, which hopefully will be studied in further research. Therefore in this study, the effect of methyl jasmonate eliciting the production of secondary metabolites on the amount of Taxol produced in the leaves and stems of *Taxus baccata* was investigated.

Materials and Methods

Plant material

A plant sample was obtained from a perennial yew (*Taxus baccata*) from the Iranian National Botanical Garden. Appropriate methyl jasmonate concentrations and elicitation time were verified based on available reports, and after preliminary testing based on the amount of Taxol produced, the appropriate case was selected. A methyl jasmonate stock solution was made and, subsequently, it was added to the pot containing yew for 48 and 72 hours, with concentrations of 0, 100, 250, and 500 μM. In this test, the pots were kept in the phytotron with 25°C, 40 to 55 percent humidity, and the light intensity was between 3 and 3.5 lux, which was constantly bright throughout the treatment. Two stem and leaf explants were used to assess the amount of Taxol produced.

Taxol extraction and HPLC analysis

The Taxol extraction was carried out in the same way as the method used by Hao et al. (2017). Dried explants following elicitation with methyl jasmonate were used for high-performance liquid chromatography. The 10 mg Taxol standard powder was dissolved in HPLC-grade acetonitrile and expanded to 100 ml to be used as a control. A sample of 5 g was mixed with 25 ml of acetonitrile, and an ultrasound was performed for 60 minutes. Then it was centrifuged for 15 minutes at 50,000 × g. The supernatant solution was filtered using a 0.45 μm filter and was ready for injection. To achieve this, about 20 μl of elicited and nonelicited extracts were injected into the HPLC device. Fractionation was performed using an Agilent 300Extend C18 column (Agilent, Santa Clara, CA, USA) with 5 μm particles, 150 mm length, and 4.6 mm internal diameter. The wavelength used for the detection of Taxol was 254 nm. The HPLC was performed at a temperature of 20°C with a flow rate of 1 ml/min. The first mobile phase consisted of methanol:water (200:800 v/v), and the second mobile phase consisted of methanol:acetonitrile (200:800 v/v) (Hao et al. 2017).

Results

Assessment of Taxol production

The HPLC method was used to estimate the amount of Taxol produced (Figs. 1 and 2). The linearity of the assay method was evaluated by Three-point standard curves. Standard calibration curves reflected good linearity of the assay in the concentration range of 2.4 to 9.6 mg/ml. The linear regression of the calibration curve produced the equation ($y = 126.28x + 133.89$), with a correlation coefficient of 0.99 (R^2) (Fig. 3).

Also, the analysis of variance for Taxol produced in the treatments indicated that all main effects, two-way interactions, and three-way interactions contributed to Taxol production because Taxol was produced in all samples (Table 1).

Examining the amount of Taxol production showed that in leaf explants, with a 48-hour duration of the treatment, Taxol production increased by increasing the concentration of methyl jasmonate from 0 to 100 μM. Then by increasing the elicitor concentration to 250 μM, we encountered a decrease in Taxol production. Finally, by applying a 500 μM methyl jasmonate concentration, Taxol production increased again. However, during the 72-hour treatment, only at the concentration of 500 μM methyl jasmonate, the amount of Taxol exceeded the control sample. In the stem explant, both in the 48-hour treatment period and in the 72-hour treatment period, only 500 μM methyl jasmonate concentration led to more Taxol production than the control sample.

The general analysis of the results showed that the highest amount of Taxol production in the samples examined in the present experiment was related to leaf explants with 48-hour treatment and methyl jasmonate with a concentration of 500 μM. The folding rate of Taxol production with three

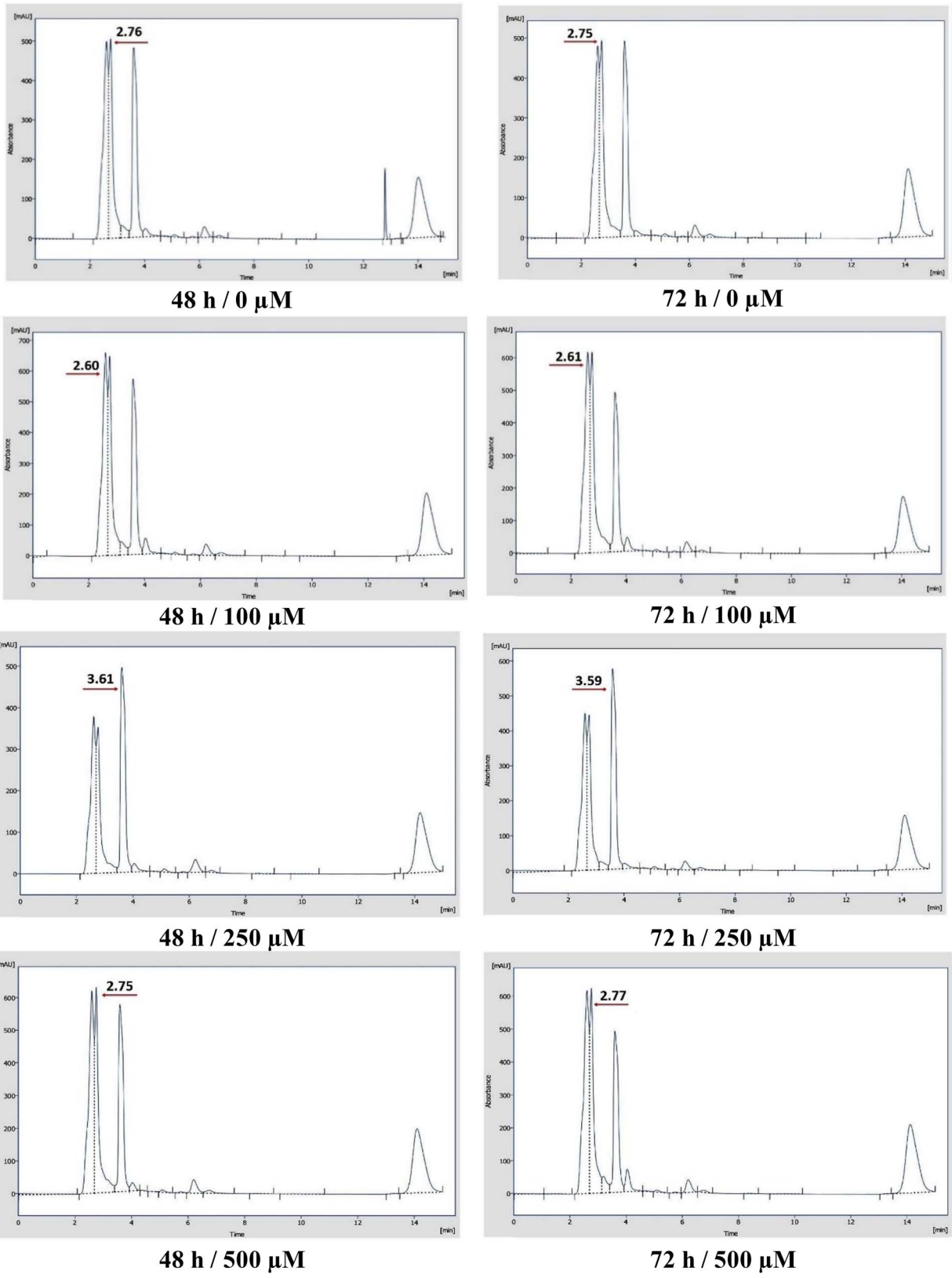

Figure 1.—Chromatograph obtained from examining the amount of Taxol produced in the leaves of the taxus plant elicited with methyl jasmonate at different times and concentrations.

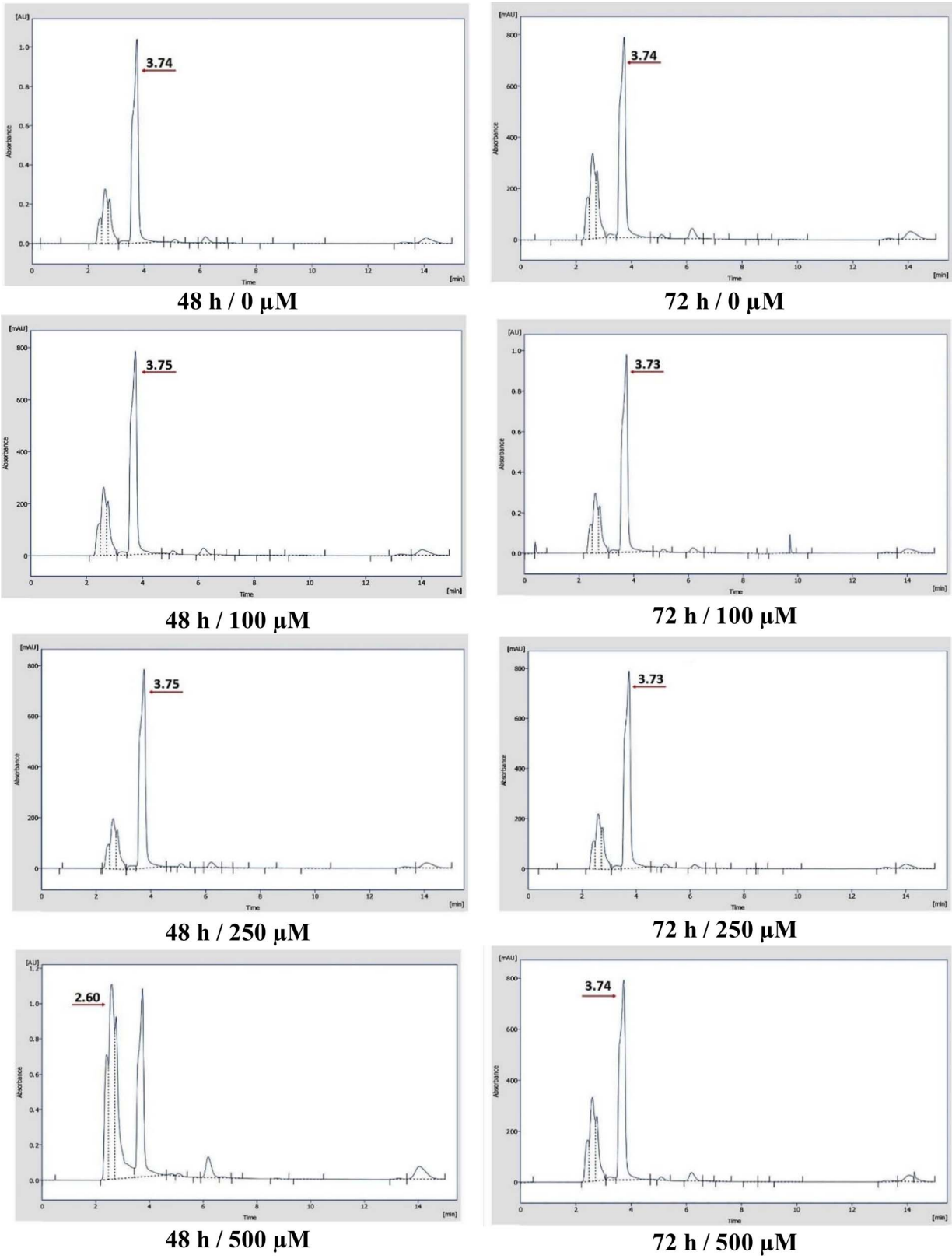

Figure 2.—Chromatograph obtained from examining the amount of Taxol produced in the stem of the taxus plant elicited with methyl jasmonate at different times and concentrations.

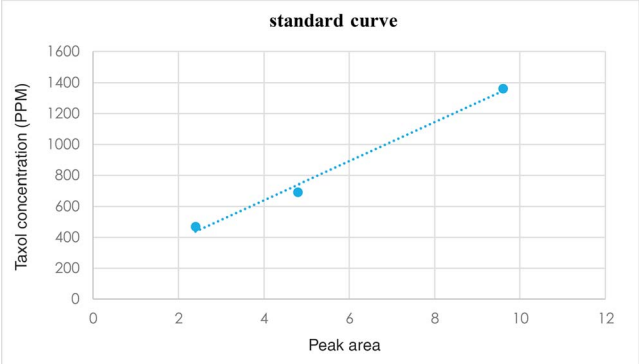

Figure 3.—The linear regression of the calibration curve for Taxol production.

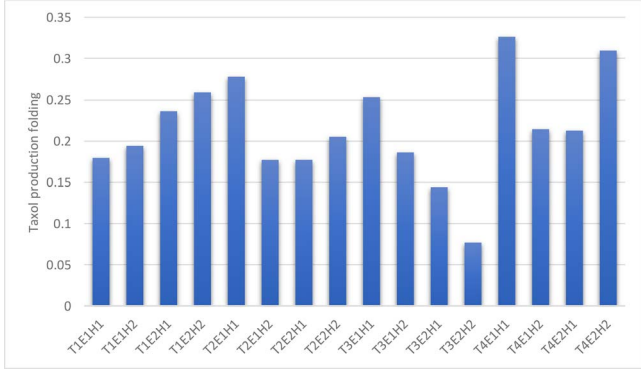

Figure 4.—The folding rate of Taxol production in leaf (E1) and stem (E2) under elicitation with different times (H1, 48 hours; H2, 72 hours) and concentrations (T1, 0; T2, 100; T3, 250; and T4, 500 M) of methyl jasmonate.

variables, explant, time, and elicitor concentration, is shown in Figure 4.

Discussion and Conclusion

The diterpenoid Taxol, obtained from various species of the genus *Taxus*, is one of the best anticancer drugs in the past decades (Vidensek et al. 1990). Jasmonates have been introduced as crucial messenger compounds in the induction process resulting in the accumulation of secondary metabolites (Yu et al. 2017). Jasmonic acid and methyl jasmonate are a new family of plant hormones commonly referred to as jasmonic acids and play a significant role in regulating the growth and development process (Gao et al. 2021). Jasmonic acid and its more active derivative, MeJA, can produce a wide range of secondary plant metabolites, such as rosmarinic acid, terpenoid indole alkaloids, etc., in different cell cultures (Almagro et al. 2014; Gao et al. 2021). JA application has been reported to produce rosmarinic acid in *Mentha piperita* (Gao et al. 2021), anthocyanin in *Vitis vinifera* (Curtin et al. 2003), and plumbagin in *Plumbago indica* roots (Gangopadhyay et al. 2011). JA and MeJA have been used as elicitors for stilbene biosynthesis in *V. vinifera* leaves (Belhadj et al. 2006), *V. vinifera* cell cultures (Tassoni et al. 2005, Taurino et al. 2015), and *Vitis rotundifolia* root cultures (Nopo-Olazabal et al. 2014). Adding MeJA to *V. vinifera* cell cultures increased anthocyanin accumulation (Tassoni et al. 2012).

In this study, the effect of methyl jasmonate on the quantity of Taxol produced in the leaves and stems of the Taxus plant was assessed using the HPLC method. The results showed that Taxol was produced in all samples elicited with methyl jasmonate. The highest level of Taxol production was in the leaves during 48 hours of elicitation with methyl jasmonate. Also, the 500 µM methyl jasmonate concentration produced the maximum amount of Taxol. The highest Taxol production was in the 48-hour elicitation with a concentration of 500 µM methyl jasmonate. The Taxol production in the plant leaves reached about 32 percent of the nonelicited sample.

Table 1.—Variance analysis of Taxol production (T, methyl jasmonate concentration; H, time; E, explant).

Source	df	Mean Square[a]
T	3	0.014
E	1	0.004
H	1	0.004
T × E	3	0.010
T × H	3	0.003
E × H	1	0.015
T × E × H	3	0.005
Total	32	
Corrected Total	31	

[a] Values were significant at $P < 0.0001$.

The effect of methyl jasmonate, ultrasonic waves, and solvent extraction was studied individually and simultaneously on the growth of specific physiological indicators and the production of Taxol in *T. chinensis* cell culture. The results showed that all treatments increased the production of Taxol and that the quantity of extracellular Taxol was more affected than the intracellular Taxol. Moreover, all treatments increased Taxol release and specific yield over the control (Asghari et al. 2012).

In one study, the effect of silver nitrate and salicylic acid elicitors was investigated individually and concurrently on the amount of Taxol production in the *T. baccata* plant by direct injection. Injection of silver nitrate and salicylic acid solutions caused a significant decrease in Taxol production compared to the control. Moreover, by increasing the concentration of elicitors, there was an additional decrease in the production of Taxol. They concluded that silver nitrate and salicylic acid solutions harmed the production of Taxol (Abbasi Kajani et al. 2012).

Another study examined the ability of isolated local endophytes of *T. baccata* to synthesize Taxol and improve its production and stability. The results demonstrated the ability of three endophytes, Acremonium, Colletotrichum, and Fusarium spp., to produce Taxol up to 116.19 µg/L. It had a significant cytotoxic effect on the human breast cancer cell line (MCF-7). A molecular assay of *Acremonium* sp. revealed the presence of the *BAPT* gene responsible for Taxol production and encoding C-13 phenylpropanoid side chain-CoA acetyltransferase. As a result, the selected endophyte has an individual metabolic system that may be activated without the host or a change in its ecology (El-Bialy and El-Bastawisy 2020).

Consequently, different elicitors have been reported that have different effects on the production of Taxol. Methyl jasmonate is one of the compounds whose positive effects in stimulating the production of secondary metabolites have been mentioned in most papers (Abdi et al. 2019,

Kowalczyk et al. 2021, Ru et al. 2022). This study evaluated methyl jasmonate as a biological elicitor, and its effect on Taxol production in *T. baccata* was evaluated. The results showed an increase in Taxol production under the effect of methyl jasmonate up to 32 percent of the nonelicited sample. Elicitation time, plant explant, and elicitor concentration were factors involved in Taxol synthesis. The highest amount of Taxol production was observed in 48-hour treatment with 500 μM methyl jasmonate concentration and the plant leaves.

Considering that we observed in our experiment that after the treatment with methyl jasmonate, the highest amount of Taxol production was in the leaf compared to the stem, it can be concluded that this higher production in the leaf itself is a significant advantage because it is not necessary to cut down thousands of yew trees every year to extract Taxol and use its stems and trunks to produce the anticancer drug Taxol. Instead, the leaf of this tree itself can act like a drug factory and produce a significant amount of Taxol every year without cutting it, saving time and money. Our best treatment (leaf explant at a concentration of 500 μM methyl jasmonate and time of 48 hours) produced 0.326 mg/g of dried leaf biomass Taxol, which is 1.81 times more than the control sample. Therefore, this method can save money and time in producing paclitaxel anticancer drugs, which is very important for drug manufacturing companies.

As a final point, this is a new experiment to understand the potential of Taxol production. In other words, more work may be needed to understand variation among trees and variation between genetic or environmental drivers.

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