Impact of phenylalanine and ultrasonic waves on the production and release of Taxol in hazelnut (Corylus avellana L.) cell culture

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Abstract

Hazelnut is one of the most important nut plants recently suggested as a sustainable source of Taxol. Taxolone is one of the most commonly prescribed chemotherapy drugs for cancer and is widely used for various types of cancer. In the present study, the effect of the phenylalanine concentration (5, 7.5 and 12.5 mg. L^{-1}) and ultrasonic waves (for 30, 45, 60 and 90 s) were investigated for their effects on cell induction and growth and Taxol and other secondary metabolite production in hazelnut cell cultures. This study used a completely randomized experimental design. The results showed that enhancing the UT for up to 45 s led to an increase in both fresh matter (Fm) and dry matter (Dm) to a certain extent, after which a decreasing trend was observed. An examination of the effects of the treatments on the accumulation of secondary metabolites revealed that phenylalanine and UT had positive effects on these indices, with the highest TFC (1.95 mg. g^{-1}) was observed in samples grown at 12.5 mg.L^{1}. Moreover, our results showed that UT had a positive impact on the production of Taxol in Hazelnut cell suspensions. Specifically, UT at 45 s had the greatest amount of Taxol (208.41 ppm) in the Hazelnut cell suspension.

Introduction

Hazelnut (*Corylus avollana* L.) has been receiving increased amounts of attention in the food industry for a long time due to its high nutritional value. The valuable seeds contained 19% protein, 60% oil, and a high percentage of vitamins B and E and mineral elements such as phosphorus. Currently, with more knowledge of this plant, it is considered for industrial and medicinal applications, with the identification and isolation of toxoids from the leaves and shells around hazelnut seeds on the one hand and the high medicinal and economic value of the metabolites on the other hand. The general treatment of cancers has opened a new horizon in the economic production of this valuable substance. In addition, this plant is also used to prepare biodiesel and bioplastics (Razavi et al. 2016).

Various factors, such as genotype, type of culture medium, type of carbohydrate, age of culture, plant growth regulators and environmental conditions, play an effective role in callus production. Research has shown the specific dependence of callus induction and plant regeneration on the type of plant and its genotype, and the induction and reproduction of callus and plant regeneration are influenced by the genotype of the plant (Han et al. 2013). On the other hand, the mechanism and extent of the effect of external regulators depend on the genotype of the plant and the amount of internal hormones (Bhaskaran and Smith 1990). However, as a general principle, for different species, cultivars within the species and different organs, a plant will not react the same to a type of tissue culture system, the control of differentiation processes depends on the growth hormones cytokinin and auxin, and the balance between these two hormones plays an essential role in the production of shoots and roots (Akbas et al. 2009).

Supplying compounds at the beginning of the biosynthesis pathway provides an excellent opportunity to increase the yield of the final product. Various biological compounds exist at the beginning or in the

middle of the biosynthesis pathway of secondary metabolites, and adding them to the culture medium can affect the production of the final product (Riedel et al. 2012). Using elicitors in cell culture is the most appropriate method for increasing productivity. Previous studies have described the enhancement of secondary metabolite production by various elicitors in medicinal plant cell cultures. Artemisinin production using elicitors increased from 1.67 mg to 2.86 mg/g of 1% dry weight (Zhan et al. 2011).

The production of secondary metabolites through cell suspension culture is widely used because of the high economic value of these compounds (Sato et al. 2001). Taxol is a diterpemoid alkaloid that belongs to a group of terpenoids called taxanes, originates from the precursor geranyl-geranyl diphosphate and is converted to taxa 4,5 (12) by the enzyme taxidine synthetase (TS). Additionally, in the final stage, it is tidylated to phenylalanine, which finally results in Naxol (Rahpeyma et al. 2015). Different strategies can be used to enhance the production of secondary metabolites in plant cell cultures (Fritz et al., 2010).

Amino acids have several major biological functions in plant cells, such as detoxification; the optimization of nutrient absorption, movement and metabolism; and the biosynthesis of vitamins (Bashir et al. 2018). In fact, amino acids are among the compounds used to produce secondary metabolites. For example, two metabolites, isoflavone and flavonoid, are derived from the amino acid phenylalanyl, which is located upstream of the metabolic pathway of phenylpropanoids. Adding phenylalanine to the culture medium increases the production of the desired metabolites (Shinde et al. 2010). The role of amino acids in the production of hyperforin and adhapyrforin in Hypericum perforatum stem culture has also been reported. The amino acids valine and isoleucine are connected to the acyl chain of hyperforin and adhapyrforin in plants. L-Isoleucine at a concentration of two millimolar induces the production of three to seven times more adapyrorin. The addition of one millimolar threonine, the precursor of isoleucine, doubles the production and accumulation of adhapyrforin (Karppinen et al. 2007).

One of the most important issues in the field of cell suspension culture is the production, increase and improvement of the quality of secondary metabolites, and considering the significant distribution and the nativeness of hazelnuts, in this study, optimization of cell culture conditions by applying phenyl amino acid treatments, alanine, ultrasonication and in vitro conditions was performed to produce the effective substance Taxol.

Materials and methods

Plant materials

The C. avollana L. seeds used in this study were provided by Ashkorat Rudsar Hazelnut Research Station. The calluses obtained from seeds were used as plant material. For this purpose, the seeds were surface disinfected with 72% ethanol for 45 seconds. After that, the seeds were washed with distilled water for two minutes and then washed again with distilled water for one minute (two steps). Subsequently, the seeds were treated with 5% sodium hypochlorite for 15 minutes and then washed with distilled water for 2 minutes (three times at one-minute intervals). For callus induction, slices were selected from the seeds

and MS media (10 mg). L^{-1} 2-4-D and 4 mg. L^{-1} BA and 2 mg. L^{-1} NAA was used. The duration of callus induction was three weeks.

Under aseptic conditions, the fragile callus was used for cell suspension culture. When the sucrose concentration was doubled along with that of the growth regulators, NAA, BA and 2,4,D were applied for improved callus growth. After proper growth of the callus and determination of the amount of cell mass produced by controlling the amount of OD in this medium and counting the live cells, in the last stage and after the cell suspension cultures reached the desired level of the OD, the treatments were performed.

The growth and viability of the cells were determined by assessing the increase in the dry weight of the cells. For this purpose, the cells were separated from the MS media and incubated at 35°C for 48 hours, after which they were weighed. One drop of suspension culture containing cells stained with one drop of 0.1% Evans blue solution was placed on the slide to determine cell viability. After 4 min, the cells were washed with distilled water and counted under a light microscope. By counting the cells that stained blue (dead cells) and the cells in which only their walls had turned blue (live cells), the percentage of viable cells was determined.

After determining the appropriate time to harvest the hazelnut cell suspension, liquid MS media containing 10 mg. L^{-1} 2-4-D and 4 mg. L^{-1} BA and 2 mg. L^{-1} NAA, phenylalanine at three concentrations (5, 7.5 and 12.5 mg/l) and ultra-sonication (UT) were applied for 30, 45, 60 and 90 s, and a control treatment (without phenylalanine and ultrasonic pretreatment) of hazelnut cell suspension was prepared (Fig. 1, 2, 3, 4). The experiment was performed in accordance with a completely randomized design with 5 repetitions. After reaching the appropriate OD, the effects of phenylalanine and UT on the amount of secondary metabolites released by hazelnut cells were investigated.

Figure 1

Cell growth curve during hazelnut cell suspension culture

Figure 2: Growth rate of hazelnut cells during the three-week growth period. (A: First week, B: second week, C: third week).

Figure 3: Treating hazelnut-grown cells (A: UT treatment at 30, 45, 60 and 90 s; B: Phenylalanine treatment at 5, 7.5 and 12.5 mg. L^{-1})

Figure 4

The linear regression equation and correlation coefficient for Taxol (n = 3).

Preparation of dry matter for measuring phenol (TPC), flavonoids (TFC), and antioxidants (TAC).

The hazelnut cell suspension was dried in an oven at 40°C for 96 hours, after which the dried material was milled. After this, 1 g of each sample was soaked in 50 ml of 80% methanol for 48 hours at room temperature. After 24 hours, the extracts were filtered through Wattman filter paper No. 4. Then, the solvent was evaporated at a temperature below 55°C. The rest of the experiments were conducted at 4°C (Pourmorad, 2006).

Total phenol content (TPC)

The total phenol content (TPC) was determined using the method of Meda et al. (2004). Gallic acid was used as a standard for generating a standard curve. The total phenol content of the extracts per mg (the equivalent of gallic acid per gram of dry weight of the plant) was reported.

Total Flavonoid Content (TFC)

To measure the TFC, 1.5 mL of methanol (80%), 100 µL of $AlCl_2$ solution (10%), 100 µL of 1 M sodium acetate solution, and 2.8 ml of distilled water were added to 500 µL of each extract. The absorbance was read after 40 min at 415 nm. Quercetin was used as a standard to construct the standard curve. The total flavonoid content of the extracts was reported in mg equivalent to quercetin per gram of DM of the plant (Mita et al. 1997).

Total Antioxidant Capacity (TAC)

Based on the DPPH method (Miliauskas et al. 2004), various concentrations (final mass ratios of extracts with DPPH were approximately 3:1, 1.5:1, and 0.75:1) of the extract were mixed with 2 mL of 0.004% methanolic solution. After 30 min at room temperature in the dark, the absorbance was read at 517 nm. The percentage of free radicals (I %) in each extract was calculated using the following formula:

I% = (A control – A sample)/A control × 100

Extraction of Taxol

According to the method of Deveci et al. (2022), the cells were dried at 45°C and ground to a fine powder. Fifty milligrams of the powder was mixed with 5 ml of methanol solution (HPLC grade) containing 60% methanol and 40% ddH_2O. The mixture was then subjected to ultrasonic treatment in a water bath for 15 min, and this process was repeated three times. The resulting mixture was centrifuged at 2000 rpm for 10 min, and the supernatant fractions were collected; this process was repeated twice. The supernatants were then evaporated with a rotary evaporator at 45°C under vacuum to obtain dry extracts. To the dry extract, 400 µL of methanol was added, and the solution was completely dissolved by vortexing. The extracts were filtered at 0.2 µm before being stored at -20°C for subsequent analysis by high-performance liquid chromatography (HPLC) (Jakabova et al. 2012).

HPLC was applied to quantify Taxol in the samples. The measurements were performed using an 18C column with a length of 50 mm and a mobile phase consisting of methanol and water at a ratio of 45 to 50 and with a flow rate of 1 mm/min. The UV wavelength used was 270 nm after calibrating the columns, and the amount of Taxol in each sample was determined by injecting a standard solution, obtaining a

calibration curve and comparing the inhibition time of the peak of pure Taxol injected with different concentrations and the peaks obtained from the injected extracts. To determine the amount of Taxol in each of the extracts, the level under the corresponding peak was measured at the desired time, and by putting this level in the equation obtained from the calibration curve, the amount of Taxol in ppm for each sample was estimated.

Results and discussion

Fresh matter (Fm) and dry matter (Dmc) of calluses

The use of UT and phenylalanine had a positive effect on increasing the fresh matter (Fm) and dry matter (Dm) of Hazelnut cell suspension samples. The results indicated that byexacerbating the UTup to 45 s led to an increase in both Fm and Dm up to a certain level, after which a decreasing trend was observed. The highest amounts of Fm (512 mg) (Fig. 5) and Dm (33 mg) (Fig. 6) were detected in Hazelnut cell suspensions treated with UT for 35 and 45 second, respectively. The 90 second UT treatment had a positive effect on this index. This was while the highest amount of this index was obtained in samples grown under the UT conditions at 90 second (Fig. 7). Additionally, our results indicate that despite the positive effect of phenylalanine on increasing Fm and Dm, the amount of Fm (Fig. 5) and Dm (Fig. 6) obtained in the samples treated with phenylalanine was lower than that in the samples treated with UT.

Figure 5

Changes in Fm (fresh matter) under the influence of phenylalanine and UT in hazelnut cell suspension culture. 30, 45, 60 and 90 indicate the UT(s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

Figure 6

Changes in Dm (dry matter) under the influence of phenylalanine and UT in hazelnut cell suspension culture. 30, 45, 60 and 90 indicate the UT(s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

Figure 7

Changes in Dm/Fm under the influence of phenylalanine and UT in hazelnut cell suspension culture. 30, 45, 60 and 90 indicate the UT(s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

Total flavonoid content (TFC), total phenolic content (TPC), and total antioxidant activity (DPPH)

Phenylalanine had an impact on the total flavonoid content (TFC) in Hazelnut cell suspensions. The cell suspensions were grown with 12.5 mg of phenylalanine. L-1 had greater TFC levels than did the control. The highest TFC level (1.95 mg. g^{-1}) was observed in samples grown at 12.5 mg.L^{1}. On the other hand, increasing the ultrasonication time decreased the amount of TFC in the studied samples. Conversely, the lowest TFC (0.519 mg. g^{-1}) was observed in callus samples grown under UT for 90 s (Fig. 8).

Figure 8

Changes in the TFC under the influence of phenylalanine and UT in hazelnut cell suspension culture. 30, 45, 60 and 90 indicate the ultrasonication time (s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

The effects of phenylalanine and UT on the total phenolic content (TPC) in Hazelnut cell suspensions were investigated, and the results were similar to those obtained for TFC; increasing the phenylalanine concentration as well as UT for up to 45 s caused an increase in the amount of TFC. The TPC at different concentrations of phenylalanine significantly increased compared with that of UT at a concentration of 12.5 mg. $L^{-}1$ phenylalanine, the TPC (10.68 mg. g^{-1}Dm) was the highest (Fig. 9).

Figure 9

Changes in the TPC under the influence of phenylalanine and UT in hazelnut cell suspension culture. 30, 45, 60 and 90 indicate the UT(s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

The total antioxidant activity (DPPH) of the Hazelnut cell suspension was positively influenced by phenylalanine and UT, as shown in Fig. 10. The DPPH radical scavenging activity increased with increasing phenylalanine concentration, up to 12.5 mg.L^{-1}. Additionally, our results showed that increasing the UT to 60 seconds had a significant effect. The highest amount of DPPH (52.25%) was obtained in the sample grown under 60 s of UT treatment (Fig. 10).

Figure 10

Changes in the TPC under the influence of phenylalanine and UT in hazelnut cell suspension cultures. 30, 45, 60 and 90 indicate the UT(s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

Taxol content in hazelnut cell suspensions

This study demonstrated that increasing the phenylalanine concentration and UT concentration had a positive impact on the production of Taxol in Hazelnut cell suspensions. Specifically, UT at 45 s had the greatest amount of Taxol (208.41 ppm) in the Hazelnut cell suspension. Our results showed that as the concentration of phenylalanine increased, the amount of Taxol added increased to a concentration of 7.5

mg. L^{-1} increased the amount of Taxol in the hazelnut cell suspension. However, this increase was lower than that in the UT treatment at 45 s (Fig. 11).

Figure 11

Changes in the Taxol concentration under the influence of phenylalanine and UT in hazelnut cell suspension cultures. 30, 45, 60 and 90 indicate the UT(s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

Heatmap of the average of Taxol accumulation in dry matter

In various studies, the results obtained from analyzing the heatmap chart are used as complementary results to express the effects of treatments on different indices (Fig. 10). The results of the heatmap of this experiment showed the positive effects of UT and phenylalanine. The effects of UT and phenylalanine on Dm/Fm were investigated in hazelnut cell suspensions. The highest correlation in this index was connected to UT at 45 s, with phenylalanine at 12.5 mg. L^{-1}. On the other hand, according to the heatmap results, the highest amount of Taxol accumulation was obtained in UT at 45 s and 7.5 mg. L^{-1}phenylalanine (Fig. 12).

Figure 12

Heatmap of the average accumulation of Taxol in the dry matter of hazelnut cell suspension cultures under phenylalanine and UT conditions. The rows show the accumulation of secondary metabolites in dry weight, and the columns show the different treatments (1, 2, 3, and 4 for the control; 30, 45, 60 and 90 s UT; and 5, 6, and 7 for 5, 7.5 and 12.5 mg/L P, respectively). A, B, C, D, E, F and G refer to Fm, Dm, Dm/Fm, TFC, TPC, DPPH and Taxol, respectively

Currently, plant cell culture is known as an alternative and renewable source for the production of secondary metabolites. The present study investigated the effects of UT and phenylalanine on the rate of Taxol release during hazelnut cell suspension culture. The application of UT and phenylalanine resulted in a change in Hazelnut cell suspension samples. However, under the UT treatment, the Hazelnut cell suspension reached its maximum growth (Figs. 5 and 6). A UT of up to 45 s led to an increase in both Fm and Dm to a certain extent. In agreement with our study, Hazrati et al. (2022) showed that the effect of UT on callus growth was positive after 1 min, but callus growth decreased with increasing exposure duration up to 3 min. It has been shown that low-intensity and low-energy UT has a range of nonlethal biological effects, which are potentially important in biotechnology. One of the most extensive nondestructive effects of these waves on living cells is an increase in membrane permeability, which enhances the absorption of compounds and the removal of intracellular products by cells. In addition, UT has been used as a stimulant for enzymatic reactions and especially secondary metabolite biosynthesis in plant cells and protoplasts (16 and 37).

Genotypes, metabolites, and elicitor applications have a great impact on determining the most appropriate method for achieving high-efficiency in vitro production of secondary metabolites. High-quality biochemical parameters are necessary for successful medicinal plant production, and a high level of phenolic compounds is normally correlated with a high ability to scavenge ROS, which is necessary to reduce the adverse effects of many stresses.

Our results showed that in Hazelnut cell suspension samples, the highest TPC and TFC were obtained under phenylalanine conditions. Among the phenylalanine treatments, 12.5 mg/L had the greatest effect on secondary metabolites. In the present study, the highest TPC and TFC in the Hazelnut cell suspension were 10.68 mg. g^{-1} and 1.95 mg. $g^{-1,}$ respectively. The bioprotection effect of amino acids has been proposed in several studies. Amino acids are a reduced form of nitrogen, and the application of reduced forms of nitrogen, such as ammonium, glycine and phenylalanine, has been shown. The use of an appropriate concentration of amino acids in coriander (*Coriandrum sativum* L.) caused an increase in the fresh and dry weights of root and leaf tissues and in the TFC and TPC compared to those of the control (Noroozlo et al. 2019). Phenylalanine is an essential amino acid for the production of phenolic and aromatic compounds in the phenylpropanoid biosynthesis pathway and plays very important structural roles in plants. The phenylalanine aminolyase enzyme is the first key enzyme involved in the biosynthesis of phenols in plant cells. According to the increasing trend of flavonoids with increasing concentrations of phenylalanine, it seems that phenylalanine is directly related to the production and accumulation of flavonoids in plants. TFC is the most abundant and the most powerful antioxidant among phenolic compounds and is present in large amounts in all parts of plants, especially in the tissues of fruits (Brouillard and Dungles 2017). Antioxidants are compounds that can effectively prevent the oxidation of macromolecules such as lipids, proteins and nucleic acids by inhibiting the initiation stage or the expansion stage of oxidation chain reactions or the production of free radicals (Sharma et al. 2012).

Our study revealed that the accumulation of Taxol under UT treatment reached its maximum level at 45 s under UT treatment. UTs can have different physiological and biological effects on plant cells depending on their exposure duration and intensity. At low energy levels, UT causes beneficial and reversible biological changes in cells and plant tissues (Hazrati et al. 2022). In previous studies, it was also reported that US at high intensities significantly reduces cell viability and survival (Safari et al. 2012; Hazrati et al. 2017). This reduction may occur due to damage caused by UT in the cell structure, including the cell membrane and wall, and organelles, which ultimately results in cell death and reduced proliferation and growth (Wu and Lin 2002). This is likely the reason for the reduction in Taxol accumulation in hazelnut cells during the 60 s and 90 s UT.

Conclusions

Considering the obtained results, phenylalanine application (7.5 and 12.5 mg. L^{-1} concentrations) enhanced TFC and TPC, and 45 and 60 s UT treatment enhanced total Taxol accumulation in hazelnut cells. Additionally, the results showed an increase in fresh and dry matter during the use of UT, and this increase can lead to favorable results in the accumulation of Taxol in hazelnut cells during cell

suspension culture. Therefore, our procedure could be valuable for further research on the biosynthesis of Taxol in cell suspension cultures of hazelnuts.

Declarations

Conflict of interest:

The authors declare that there are no conflicts of interest, financial or otherwise, in this study.

Author Contribution

this manuscript is a results of a Ph.D thesis so, all Authors had contributions during laboratory experiment, data analysis and manuscript preparation.

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Figures

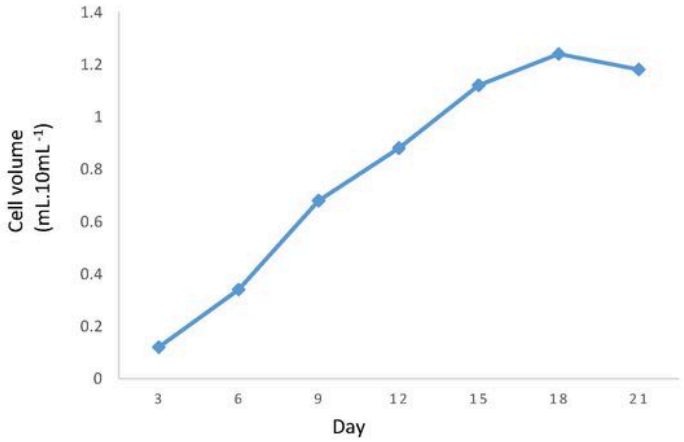

Figure 1

Cell growth curve during hazelnut cell suspension culture

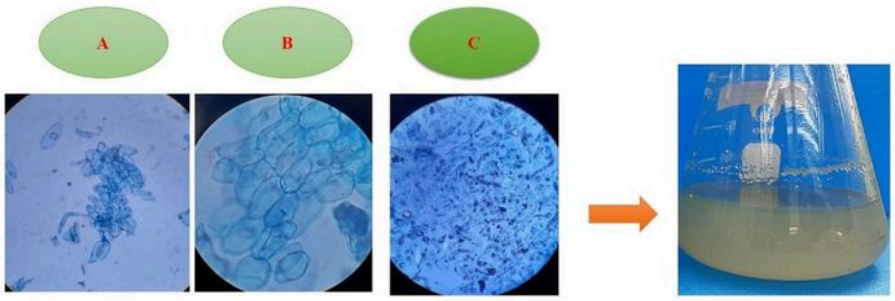

Figure 2

Growthrate of hazelnut cells during the three-week growth period. (A: First week, B: second week, C: third week).

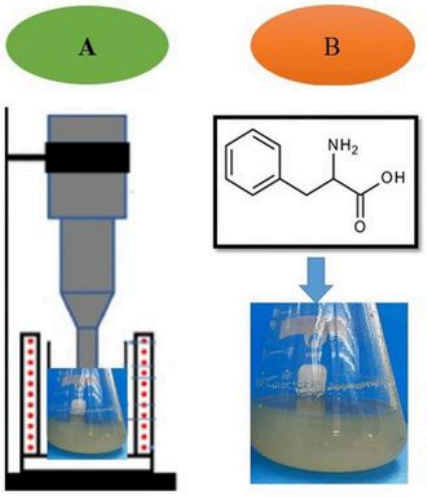

Figure 3

Treating hazelnut-grown cells (A: UT treatment at 30, 45, 60 and 90 s; B: Phenylalanine treatment at 5, 7.5 and 12.5 mg. L^{-1})

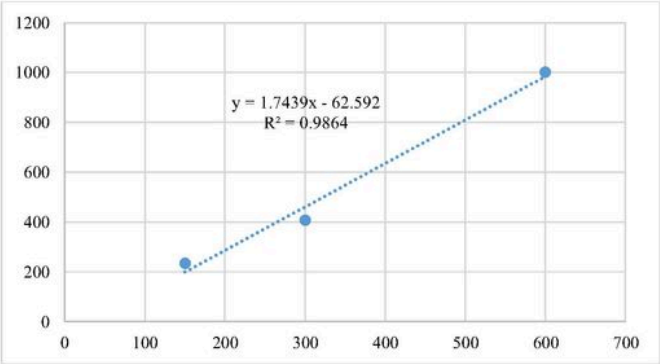

Figure 4

The linear regression equation and correlation coefficient for Taxol (n=3).

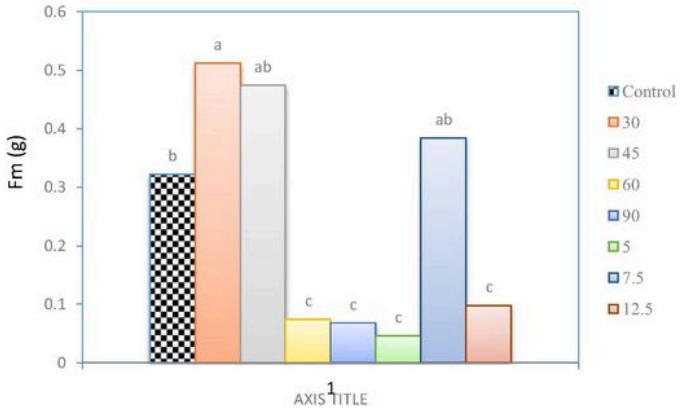

Figure 5

Changes in Fm (fresh matter) under the influence of phenylalanine and UT in hazelnut cell suspension culture. 30, 45, 60 and 90 indicate the UT(s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

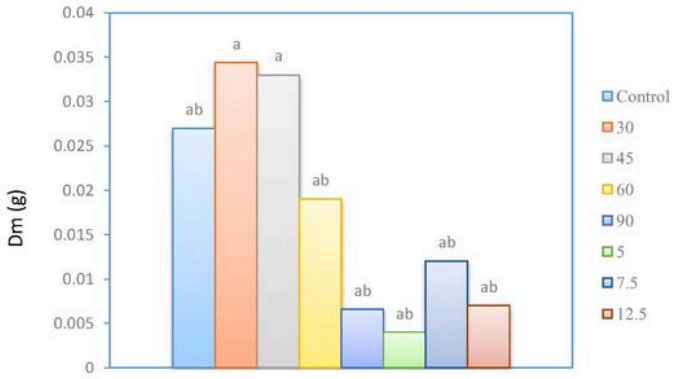

Figure 6

Changes in Dm (dry matter) under the influence of phenylalanine and UT in hazelnut cell suspension culture. 30, 45, 60 and 90 indicate the UT(s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

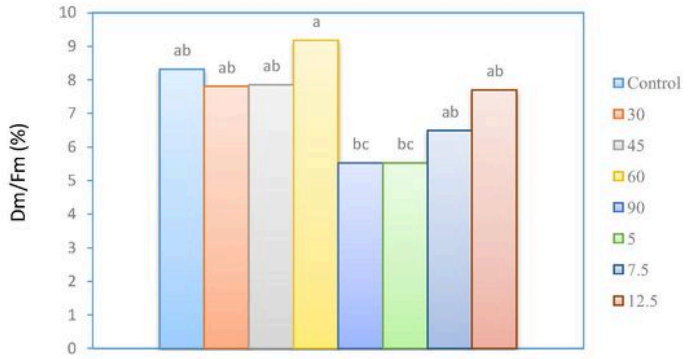

Figure 7

Changes in Dm/Fm under the influence of phenylalanine and UT in hazelnut cell suspension culture. 30, 45, 60 and 90 indicate the UT(s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

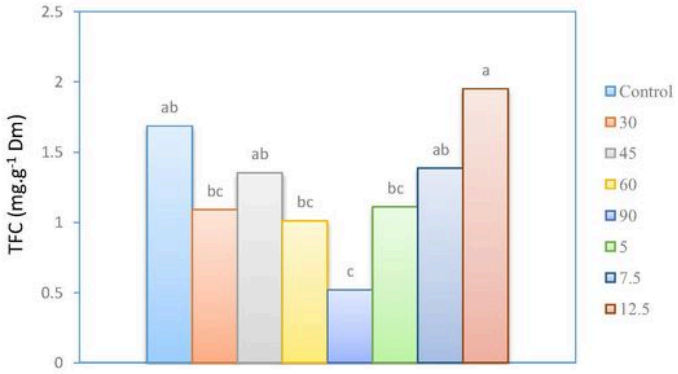

Figure 8

Changes in the TFC under the influence of phenylalanine and UT in hazelnut cell suspension culture. 30, 45, 60 and 90 indicate the ultrasonication time (s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

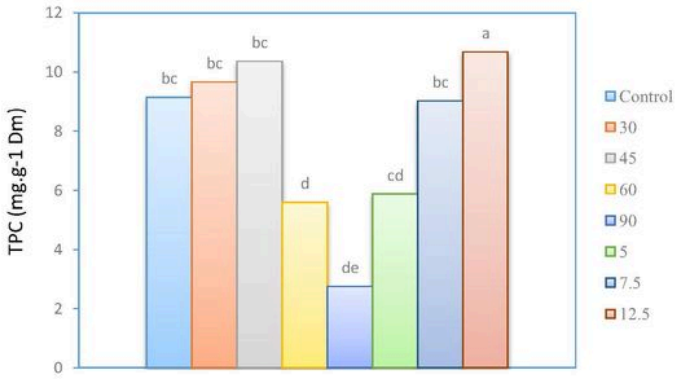

Figure 9

Changes in the TPC under the influence of phenylalanine and UT in hazelnut cell suspension culture. 30, 45, 60 and 90 indicate the UT(s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

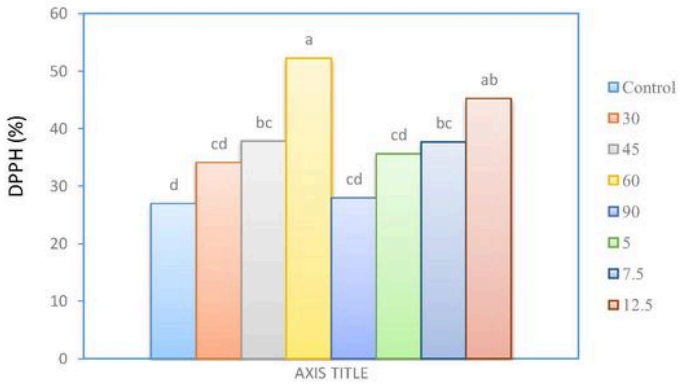

Figure 10

Changes in the TPC under the influence of phenylalanine and UT in hazelnut cell suspension cultures. 30, 45, 60 and 90 indicate the UT(s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

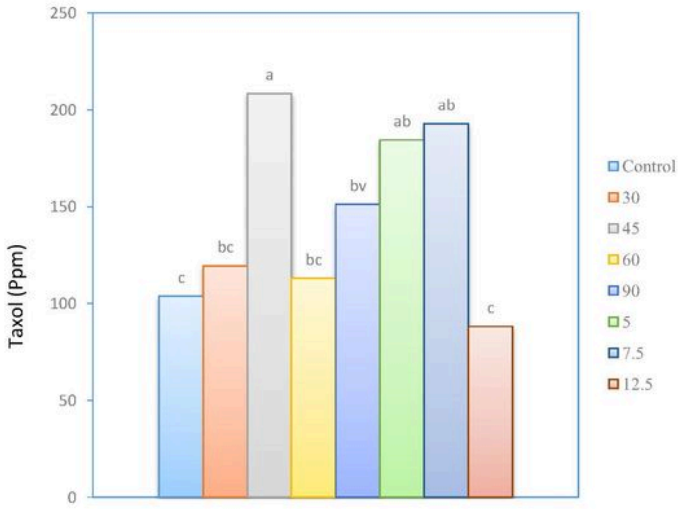

Figure 11

Changes in the Taxol concentration under the influence of phenylalanine and UT in hazelnut cell suspension cultures. 30, 45, 60 and 90 indicate the UT(s). 5, 7.5 and 12.5, indicating the concentration of phenylalanine (mg. L^{-1}), respectively.

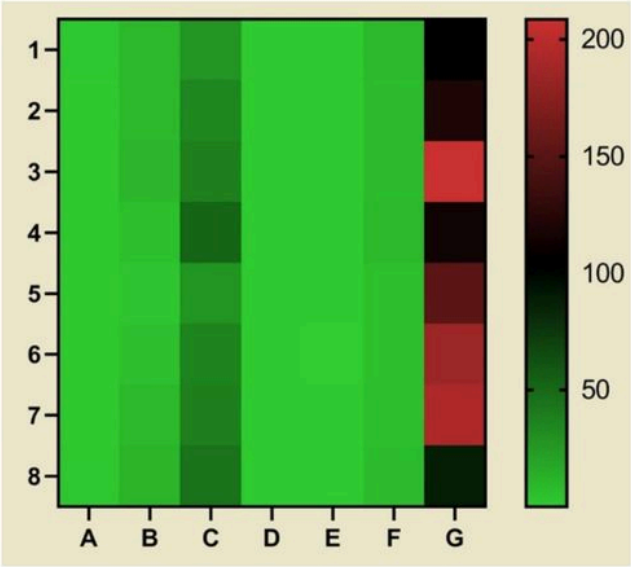

Figure 12

Heatmap of the average accumulation of Taxol in the dry matter of hazelnut cell suspension cultures under phenylalanine and UT conditions. The rows show the accumulation of secondary metabolites in dry weight, and the columns show the different treatments (1, 2, 3, and 4 for thecontrol;30, 45, 60 and 90 s UT; and 5, 6, and 7 for5, 7.5 and 12.5 mg/L P, respectively). A, B, C, D, E, F and G referto Fm, Dm, Dm/Fm, TFC, TPC, DPPH and Taxol, respectively