Investigation of callus weight, total phenol, and total flavonoid contents in four different tissue types of *Taxus baccata* L.

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Abstract

Taxus baccata belongs to the Taxaceae family. Taxol is one of the most important natural anti-cancer compounds in the world, which is obtained from different taxus species. But due to the very small concentrations of this drug in the plant, suitable alternative methods should be used to produce this drug from the plant. Plant tissue callus and cell culture can be one of the most effective methods for producing the anticancer drug Taxol. In this research, callus formation and phenolic and flavonoid compounds of four different types of *Taxus baccata* L. tissues (stem, leaf, shoot tip and leaf + node) were investigated. For the induction of callus, four types of explants were placed in Gemburg culture medium with 6 mg/L 2,4-D, 0.5 mg/L kinetin and 30 g/L sucrose. After 4 weeks, the fresh weight and dry weight of callus, total phenols, and total flavonoids were measured in four types of tissue. The results showed that the highest fresh and dry weight of callus belonged to the shoot tip tissue while the lowest fresh and dry weight of callus belonged to the leaf tissue. Also, the total phenol and flavonoid contents of the four tissues under study showed that the lowest values of total phenol and total flavonoid belonged to the shoot tip tissue and other tissues were not different from each other.

Keyword: *Taxus baccata*, Taxol, callus weight, total phenol, total flavonoids

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Introduction

Taxus baccata belongs to the genus Taxus, which is a slow-growing and shade-loving coniferous tree. The plant, also known as yew, is scattered in humid and semi-humid areas, has little natural reproduction, and is of high industrial and medicinal importance ((M Pirali, 2005; Omidi et al., 2011). Yew has lost its natural habitats due to various ecological factors, including climate warming and destruction by human factors, and is on the verge of extinction. These conditions have caused this valuable plant to be under threat (Rahmati et al., 2017). Therefore, propagation through tissue culture can be an effective way in reviving this valuable species. On the other hand, this tree is the primary source for the production of the anti-cancer drug Taxol, which is used to treat many cancers, including breast, uterus, lung, bladder, etc. (Caruso et al., 2000; Kajani et al., 2010). Taxol is a diterpenoid compound that is obtained from different species of yew and its

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scientific name is paclitaxel ((Mihaljevic et al., 2002; Nicolaou et al., 1996). Taxol production from cultured yew calluses paved the way for subsequent research in this field (Christen et al., 1989). The anti-cancer agent Taxol is present in all parts of the yew tree except its fruit, even though its concentration is very low varying in different species of yew from 0.01 to 0.03 mg per gram of dry tissue (Escrich et al., 2023). To prepare one gram of Taxol, it is necessary to cut three-hundred-year-old mature trees (Imamura et al., 2023). In fact, yew tree is the most important source of Taxol, and owing to the importance and the wide and increasing acceptance of this substance, various species of yew, including *Taxus baccata*, have received much attention (Imamura et al., 2023) while cutting of trees for Taxol production has put this species at risk of extinction (Tandoğan et al., 2023). Also, the production of Taxol from plant material is not cost effective (Asgharzadeh et al., 2023). For this reason, there is a search around the world to find alternative methods for Taxol production that ensure producing more Taxol while saving this valuable specie(Zhang et al., 2023).

The chemical structure of Taxol is very complex and more than 20 enzymes are involved in its biosynthetic pathway. Because of the complexity of the biosynthetic pathway, its synthetic production is very difficult (Zhao et al., 2023). An alternative method of Taxol production is the use of tissue and cell culture technology (Zhao et al., 2023). Callus is an undifferentiated and amorphous mass of cells that can be obtained from different plant tissues. This cell mass is considered a good source for the production of secondary metabolites, including Taxol owing to its rapid growth and proliferation (Liu et al., 2023). Also, in addition to producing secondary metabolites from callus, callus can be used for regeneration and propagation of plants, including different species of taxus (Ozyigit et al., 2023). The origin of suspension culture is from callus, so for the commercial production of Taxol, yew cell suspension culture is highly important. Schiff et al. (1974) cultivated taxus callus for the first time(Mohamed et al., 2023). The stems of different species of taxus were used to induce callus and their fresh weight was tested in Gamborg's B5 and MS culture medium. Gamborg's B5 culture medium was found to increase callus growth better compared to MS culture medium, and taxus cell suspension culture was prepared with the obtained calluses (Tomilova et al., 2023).

The concentration of Taxol varies in different species of taxus, different organs of most species of taxus, with factors such as the age and gender of the plant, climatic and weather conditions, soil condition, and the light condition (Shahrajabian et al., 2023). Delavar (1998) compared the concentration of Taxol in different organs of Taxus baccata in two regions of Gorgan and Noor according to seasonal changes and recorded the highest concentration of Taxol in leaves (between 0.0285 and 0.055% of dry weight), roots (0.023 to 0.037% of dry weight), and the bark of young stems, in that order. In another study by Ghorbanli and Delavar (2001), concentration of Taxol in stem tissue (0.021 to 0.056% of dry weight) was higher than that of leaves (0.018 to 0.032% of dry weight). Furthermore, the Taxol contents of the callus culture and cell suspension of *T. baccata* and *T. berevifolia* species were investigated and the results showed that *T. baccata* species has the potential to produce this metabolite more than *T. berevifolia* species (Ahadi et al., 2013). The present study aimed at comparing phenolic compounds in the callus of four tissue types, namely stem, leaf, shoot tip, and leaf + node of *Taxus baccata* species.

Materials and Methods

T. baccata seedlings (4-6 years old) were purchased from Tonekabon and kept in the Research Greenhouse of Zanjan University. After the emergence of new branches, they were used for explants, and 7-8 cm pieces were cut from the young branches and transferred to the tissue culture laboratory as explants. The explants were placed under running water for disinfection for one hour, transferred under Lamin Air Flow to continue the disinfection process, placed in 70% ethanol for 30 seconds, then in 2% sodium hypochlorite for 10 minutes, and washed three times with sterile distilled water. In the last step, the explants were placed in 0.1% mercury chloride for 8-10 minutes and then washed three times

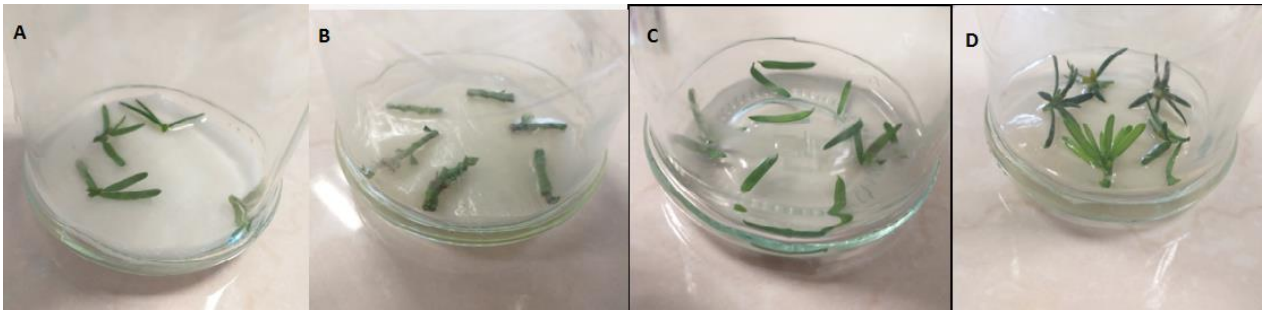

Fig. I. Four different tissue types of *Taxus baccata* L. for callus induction; A: leaf + node, B: stem, C: leaf, D: shoot tip

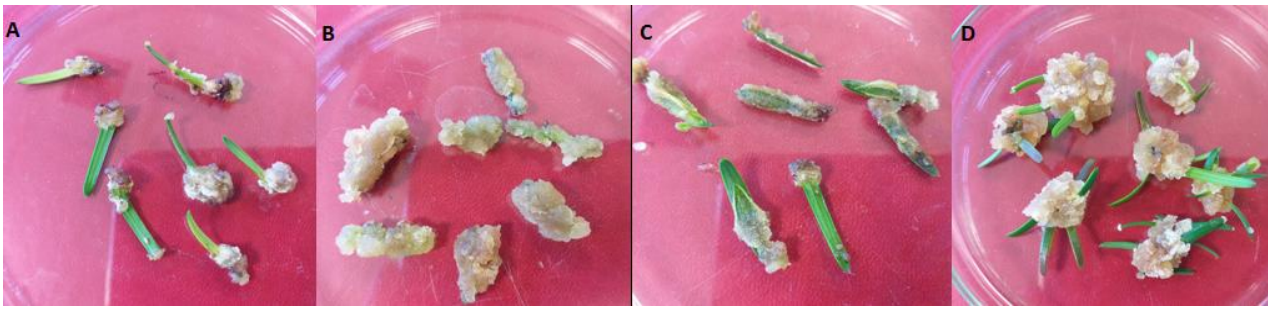

Fig. II. Callus induction in four different tissue types; A: leaf + node, B: stem, C: leaf, D: shoot tip

with sterile distilled water. After the disinfection, the four tissues of the explants (leaf, stem, shoot tip, and leaf + node were separated (Fig. I).

To induce callus (Fig. II), Gamborg (B_5) culture medium containing 6 mg/L of 2-4 dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L of Kinetin (KIN) with 30 g/L of sucrose (pH 5.7) was used (Gamborg et al., 1968). After cutting, each explant was placed horizontally on a separate liquid culture medium and kept in a growth chamber at 25 ℃ in the dark. The explants were analyzed After 4 weeks.

Fresh and dry weights of callus assays

The calluses were separated from the tissues after 4 weeks, and their fresh weight was measured with a digital scale model (CANDGL300). Dry weights were also measured using a digital scale model (CANDGL300) after the calluses were dried in an oven at a 65 ℃ for 48 hours.

Total phenols assays

To prepare methanolic extract, 30 mg of dried callus was weighed, sonicated well, and then centrifuged for 10 minutes at 5000 rpm.The measurement of total phenols was carried out by Folin Ciocalteu method (Norani et al., 2023). For this purpose, 1900 microliters of distilled water plus 100 microliters of methanolic extract and 500 microliters of 10% Folin Ciocalteu were put into a Falcon, sonicated well, kept for 5 minutes, and were added 500 microliters of 7% sodium carbonate. The solution was then put in the dark and room temperature for 30 minutes, and the absorbance was read at the wavelength of 765 nm. For the control sample, 80% methanol was used instead of the extract. Gallic acid was used as the standard to draw the calibration curve for total phenol. For this purpose, a base solution of 1000 ppm was first prepared. According to the absorbed wavelength range of the samples, concentrations (5, 10, 50, 100, 200, 400, 600, and 700 mg/l) were prepared and their absorption was read using a spectrophotometer (Specorp 250 Jena-History) at a wavelength of 765 nm. Then, the equation of the determination line and the approximate values of the samples were calculated.

Measurement of total flavonoid

To measure the total flavonoid content, aluminum chloride method was used (Perak Junaković et al., 2023). For this purpose, 2700 microliters of distilled water along with 100 microliters of methanolic extract, 100 microliters of 10% aluminum chloride, and 100 microliters of 1 M

potassium acetate were poured into a falcon tube and kept for 30 minutes in the dark and at room temperature; then, the absorbance was read in the wavelength 415 nm.

For control, 80% methanol was used instead of the extract. Quercetin standard was used to draw the flavonoid calibration curve. For this purpose, a base standard solution with a concentration of 1000 ppm was prepared. Then, according to the range of wavelengths absorbed by the samples, concentrations of (5, 10, 50, 100, 200, 400, 600, and 700 mg/l) were prepared, and the absorbance of the samples was measured. Zero concentration was considered as a control and the calibration graph was calculated using the equation of the line obtained from the read absorbance.

Statistical Analysis

The experiments were conducted in a completely randomized design. To test the normal distribution of the observed data, Shapirowilk test was used. One-way analysis of variance (One-way ANOVA) was used to compare the results. To determine the significant difference, Duncan test was used. Statistical analysis was performed using Spss Version 26 software ($p<0.5$), and charts were drawn using Excel 2013.

Result

Fresh and dry weights

The results of Duncan's test revealed a significant difference amongst tissues in terms of callus fresh weight. As Fig. (III) shows, a significant difference was observed amongst the tissues, so that the highest and lowest fresh weights were related to the shoot tip and leaf tissues, respectively. The shoot tip showed the highest callus fresh weigh, followed by the stem and leaf + node tissue, respectively. The lowest fresh weight of callus was related to leaf tissue.

Similar to the fresh weight of callus, the dry weight of calluses in four types of tissues were significant ($p<0.01$) according to the one-way ANOVA. Also, running the Duncan's test revealed a significant difference between the tissues with the shoot tips having the highest dry weight of callus, followed by the stem, and then the leaf + node. On the other hand, the lowest dry weight of callus was related to the leaf samples (Fig. IV).

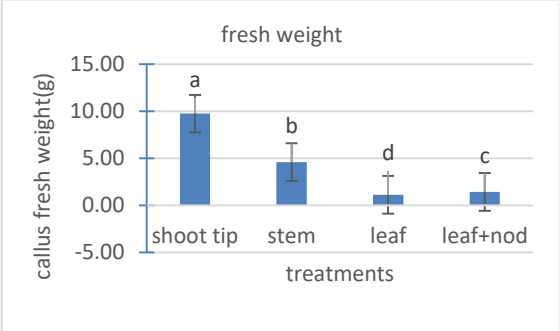

Fig. III. Fresh weight of four different tissue types (leaf, stem, shoot tip, and leaf + node)

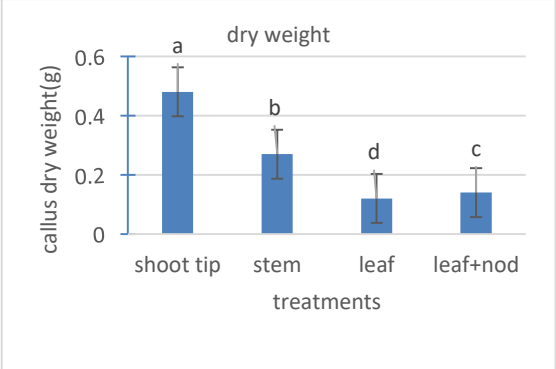

Fig. IV. Dry weight of four different types of tissue (leaf, stem, shoot tip, and leaf + node)

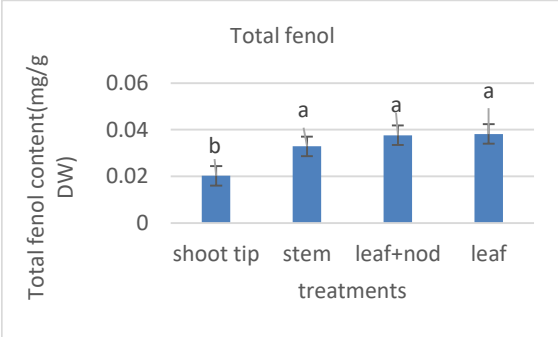

Fig. V. Total phenol of four different types of tissue (leaf, stem, shoot tip, and leaf + node)

Total phenolic contents

Examining total phenol contents in the four tissues showed differences amongst them. However, the results of Duncan's test revealed that only the shoot tips were significantly different from the other tissues, and total phenols were lower than other tissues. Also, the other three tissues of the stem, leaf, and leaf + node were not significantly different in this attribute from each other (Fig. V).

Total flavonoid contents

A difference was observed in the concentration of total flavonoids among the tissues under study. By performing Duncan's test, it was found that shoot tips were significantly different from the rest of the tissues in terms of flavonoid contents. Total flavonoid content of the shoot tips was lower than the other tissues while the stem, leaf, and leaf + node tissues were not significantly different from each other (Fig. VI).

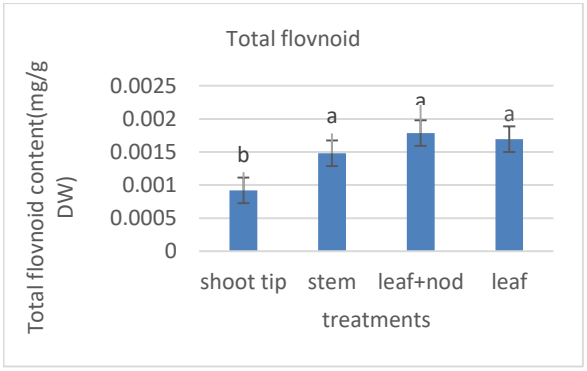

Fig. VI. Total flavonoid contents of four different types of tissue leaf, stem, shoot tip, and leaf + node

Discussion

In this research, a comparison was made between different tissues of the yew plant in terms of fresh and dry weight of callus as well as phenolic and flavonoid compounds. Appropriate concentrations of Auxin (2-4-D) and cytokinin (kinetin) hormones were used to induce callus for all tissues. Callus production depends on the amount of growth hormones used, and the balance between auxin and cytokinin hormones is a determining and important factor (Kajani et al., 2010). In this research, the same level of hormones was used for all tissues, and the only factor was the type of explant to assay callus formation in terms of fresh and dry weights as well as the concentration of phenolic and flavonoid compounds in shoot tip, stem, leaf, and leaf + node.

Comparison of the wet and dry weights of the four different tissue types showed differences amongst them. The maximum and minimum wet and dry weights of callus was recorded in shoot tip and leaf samples, respectively. After the shoot tips, stem samples had the highest wet and dry weights of callus. Considering that the maximum fresh and dry weights of callus were recorded in the shoot tip tissue, it is possible that the accumulation of plant hormones in the shoot tip tissue might be more than the other tissues, as they can increase the callus formation rate in the tissue.

The type of explant can affect callus formation in tissue. Moreover, choosing an explant at the optimal growth stage plays a key role in the success of tissue culture in vitro (Çöçü et al., 2004). The morphological complexity of an explant along with the selection of suitable plant growth regulators has a significant effect on callus induction (Çöçü et al., 2004).

In this research, for the first time, the amount of total phenol and flavonoid in the callus of four different tissue types of a yew species (*T. baccata*) was determined. Total phenol contents of the tissue of the shoot tips in this study was lower than other tissues, and there was no significant difference amongst the other tissues in the concentration of total phenols. Similarly, total flavonoid contents of the shoot tip tissues were significantly lower than other tissues, and the other tissues were not significantly different in this attribute. The stem tip tissue is the locus of meristem cells, where growth hormones accumulate. Therefore, this tissue has the ability to produce more callus. Guleria et al. (Guleria et al., 2013) recorded the total phenol content of 69.96 ± 2.73 mg GAE/g in the methanolic extract of the Indian *Taxus baccata.* In another study, the total phenolic and flavonoid contents of the methanolic extracts of Serbian *Taxus baccata* were found as 92.13 ± 0.84 mg GAE/g dry extract and 161.98 ± 1.02 mg RE/g dry extract, respectively (Milutinović et al., 2015).

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