Investigating callus induction and quality in four different types of Common yew (*Taxus baccata* L.) tissues

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Abstract

Taxus baccata plant is one of the most important sources of the anti-cancer drug Taxol. This plant is among the endangered species due to its low growth and reproduction, as well as excessive harvesting from nature. The amount of this valuable drug in the plant is very low. Therefore, by using the tissue culture technique, the approach of exploiting this valuable plant to produce Taxol can be changed from natural areas to controlled conditions, thus saving it from the danger of extinction and allowing for the extraction of more of this medicine from the plant. In this research, four different types of yew tissue (stem, leaf, shoot tip, and leaf + node) were investigated. For the induction of callus, four types of explants were placed in Gamborg culture medium with 6 mg/L 2,4-D, 0.5 mg/L kinetin, and 30 g/L sucrose. After four weeks, callus fresh and dry weight, callus formation rate, callus browning rate, and callus size were investigated. The results showed that the highest fresh and dry weight of callus belonged to the shoot tip tissue, and the lowest fresh and dry weight of callus belonged to the leaf tissue. Also, stem tissue and leaf + node tissue showed the highest percentage of callus formation (100%). The results of callus browning showed that the stem tissue browned more than other tissues. Examination of the callus size showed that the shoot tip tissue had the largest callus size compared to other tissues.

Keywords: Common yew (*Taxus baccata* L.), Taxol, callus induction, callus browning, callus size.

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Introduction

Common yew is a coniferous, evergreen tree belonging to the family Taxaceae and the genus *Taxus* (Thakur and Kanwal, 2024). All yew species are considered endangered plants (Iqbal et al., 2020). Yew has slow growth and is one of the shade-loving plants scattered in humid and semi-humid areas (Ahmed et al., 2021; Hematzadeh et al., 2023). Although yew tolerates low light, it shows better growth under higher light conditions (Iszkulo, 2010; Wu et al., 2023).

In the Arsbaran forests, the red oak is considered a key species (Ghanbari and Kern, 2021). The wood of the yew tree is widely used, including in

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industries and pharmaceuticals. Various ecological factors, such as global warming, human-induced degradation, and its indiscriminate use, have contributed to the endangerment of this valuable species(Halder and Jha, 2023).

Yew leaves and bark are used to produce the anti-cancer drug taxol (generic name: paclitaxel) (Yan-Hua et al., 2020). All parts of the yew tree, except its red fruit, contain alkaloids, diterpenoid compounds, ligands, resin, and tannins, contributing to the plant's toxicity (Yan-Hua et al., 2020). Taxol, a secondary metabolite, is produced in all *Taxus* species (Tomilova et al., 2023).

Taxol was first isolated from the bark of *Taxus brevifolia* (Suffness and Wall, 2021) and is regarded as the most valuable anti-cancer drug (Sinha, 2020). Among *Taxus* species, *T. chinensis, T. brevifolia,* and *T. baccata* produce the highest quantities of taxol (Tomilova et al., 2023). Over the last two decades, extensive research has focused on the chemical synthesis (Kar et al., 2021)and cell suspension cultures of various *Taxus* species (Kochkin et al., 2023).

Taxol is used to treat various cancers, including lung, bladder, breast, and uterine cancers, and was first utilized in 1977 (Kochkin et al., 2023; Srivastava and Tiwari, 2022). The U.S. Department of Agriculture has recognized paclitaxel from *Taxus brevifolia* as a vital anti-cancer drug (Cech and Oberlies, 2023). Taxol is among the most profitable anti-cancer drugs (Wang et al., 2022).

Producing 1 Kg of taxol requires 10,000 Kgs of yew bark or 3,000 yew trees, while treating one cancer patient requires 2.5 to 3 grams of taxol (Shao, 2024). Thus, sourcing taxol from plants risks depleting these species and leading to their extinction (Frisvold, 2023). Consequently, research has turned to alternative taxol production methods due to increasing demand.

The chemical synthesis of taxol is challenging and costly due to its complex biosynthetic pathway (Sabzehzari et al., 2020). Alternative methods such as tissue culture, cell suspension culture, endophytic fungi culture, and metabolic engineering are being explored(Wawrosch and Zotchev, 2021).

Callus cultivation plays a significant role in biotechnology for producing plant products. Callus refers to the growth of undifferentiated plant tissue and is used in pharmaceuticals, food, and agriculture (Karabulut et al., 2024). The metabolites produced by callus culture are diverse and treat various diseases (Hassanpour, 2024; Sajeev et al., 2024).

Callus formation typically begins with selecting plant species rich in desired compounds. Industrial-scale production can employ callus cells via bioreactors (Bapat et al., 2023). Optimizing conditions such as light, temperature, humidity, and nutrients is critical for successful callus formation (Sidik et al., 2024). Biotic and abiotic stimuli trigger callus formation, with a balanced ratio of auxins and cytokinins being essential (Mai et al., 2024). Callus culture is also beneficial for conserving endangered species by eliminating the need for extensive plant harvesting (Rather et al., 2022).

Recent advancements focus on optimizing callus culture techniques to maximize secondary metabolite production (Selwal et al., 2023). For example, *Taxus baccata* callus production has been explored using various tissue types and culture media to enhance growth and metabolite yield (Ghasemnezhad et al., 2024). This approach offers a promising solution to revive endangered species like *Taxus baccata* and meet the growing demand for taxol.

According to the World Health Organization, cancer was the second leading cause of death worldwide in 2018 (Li and He, 2022). As a key natural anti-cancer agent, paclitaxel's demand has driven the development of tissue culture techniques since the 1990s (Liu et al., 2023). Recent advancements emphasize optimizing culture media, precursor nutrition, and elicitors to enhance taxol production(Perez-Matas et al., 2023).

The purpose of this experiment was to investigate and compare the potential of four different tissue

types of *Taxus baccata* L. in terms of callus induction, morphological traits, and browning levels, to identify the most suitable explant for successful callus culture and conservation of this endangered species.

Materials and Methods

Explant Preparation

Young yew branches from the greenhouse at Zanjan University were used as explants. After the shoots grew and new shoots appeared, 5–7 cm of the newly grown shoots were cut and transferred to the plant tissue culture laboratory.

Sterilization of Explants

The explants were placed under running water for one hour, then disinfected under a laminar hood for 30 seconds in 70% ethanol, followed by treatment in 2% sodium hypochlorite for 10–12 minutes. Afterward, the explants were washed three times with sterile distilled water. In the final step, the explants were sterilized with 0.1% mercury chloride for 8–10 minutes, followed by another wash with sterile distilled water. After the disinfection process, the explants were cut, and the leaf, shoot tip, stem, and leaf + node tissues were separated. The isolated tissues were cultured separately in callus induction medium.

Cultivation of Explants for Callus Induction

After sterilization, the explants were cultured in a medium containing growth-regulating hormones to induce callus. For callus induction, Gamborg (B5) culture medium (Gamborg et al., 1968) was used, containing 6 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l of kinetin (Kin), along with 30 g/l of sucrose and a pH of 5.7. After cutting, the explants were placed separately, horizontally, on the liquid culture medium (Fig. I) and kept in a growth chamber at 25 °C in the dark. Thirty samples of each tissue type were cultured to obtain callus. After 4 weeks, the explants were analyzed.

Fresh and Dry Weight of Callus Assays

After 4 weeks, the calluses were separated from the culture medium, and their fresh weight was immediately measured using a digital scale (model: candgl300). Then, the calluses were dried for 48 hours in an oven at 65°C, and their dry weight was measured using the same digital scale (model: candgl300) and recorded (Fig. II)

Callus Browning Assays

To determine the amount of browning, after 4 weeks, the browning was classified into 5 levels (Khosroushahi et al., 2011). Number 0 was assigned to callus that did not turn brown, Number 1 for a low amount of browning, Number 2 for between low and medium browning, Number 3 for medium browning, Number 4 for between medium and high browning, and the maximum amount of browning was assigned to number 5. For each tissue, all 30 separate samples were numbered according to the level of callus browning.

Callus Size Assays

To measure the callus size after 4 weeks, the callus size was classified into 5 levels (Khosroushahi et al., 2011). No growth of callus was assigned number 0, and the largest callus size was assigned number 5. For each tissue, all 30 separate samples were numbered according to callus size.

Percentage of Callus Formation Assays

Callus induction percentage was calculated using the following formula (Alonso-Herrada et al., 2016):

$$CI = n / N \times 100$$

Where:
CI = Callus induction percentage
n = Number of explants that have formed calluses
N = Total number of cultured explants

Statistical Analysis

The experiments were conducted in a completely randomized design. The results were analyzed using SPSS software, version 26. Data analysis was done through the Non-Parametric Test and

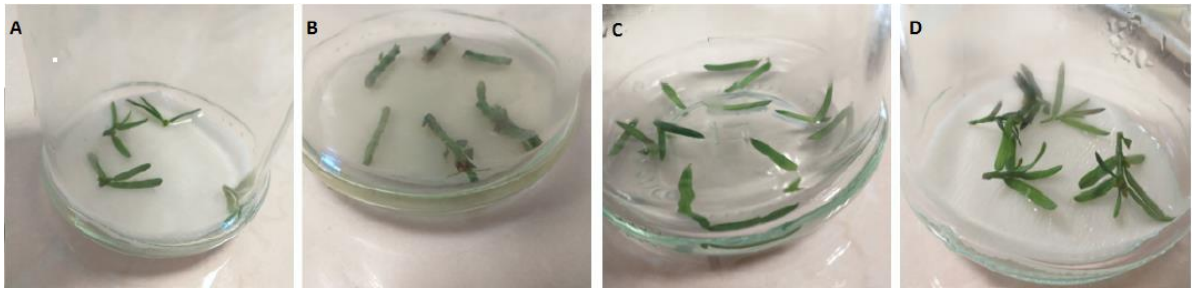

Fig. I. Four different tissue types of *Taxus baccata* for callus induction A (leaf + node), B(stem), C(leaf), D (shoot tip).

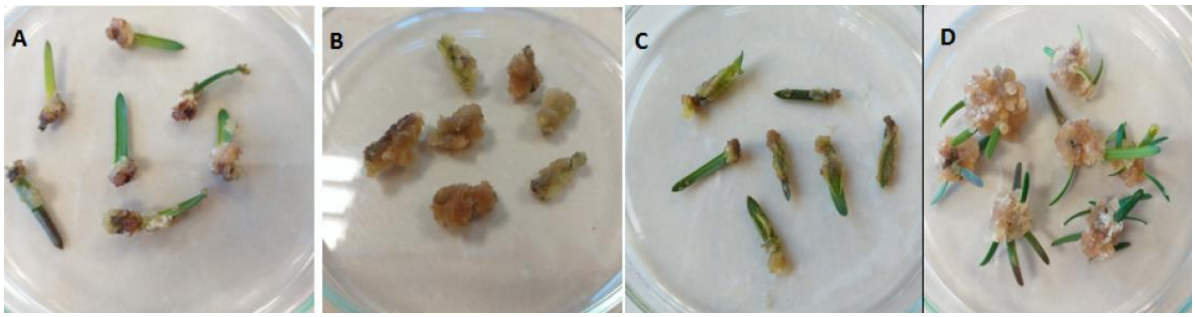

Fig. II. Induced calluses in four different tissue types A (leaf + node), B(stem), C(leaf), D (shoot tip) .

independent samples. To determine the significant difference, the Duncan test was used. The graphs were also created using Microsoft Office Excel 2013.

Results

Fresh and Dry Weights

After performing the test for normality and checking the normality of the data, the results indicated that the use of four different types of tissue led to significant differences. The results of Duncan's test revealed a significant difference between the tissues in the amount of callus fresh weight. As shown in the figure related to fresh weight (Fig. III), a significant difference can be observed between different tissues, with the highest fresh weight observed in the shoot tip tissue and the lowest fresh weight in the leaf tissue. The shoot tip tissue exhibited the highest amount of callus fresh weight. Following the shoot tip tissue, the stem tissue showed the second highest amount of callus fresh weight. The leaf + node tissue came next, with the lowest amount of callus fresh weight observed in the leaf tissue.

Similarly, the results obtained for the dry weight of callus from the four tissue types were also significant at the 1% level ($p < 0.01$) according to the one-way ANOVA analysis. Duncan's test revealed that there is a significant difference between the tissues, with the shoot tip tissue having the highest dry weight of callus, followed by the stem tissue, the leaf + node tissue, and, finally, the lowest dry weight of callus being related to the leaf tissue (Fig. IV).

Callus Formation Percentage

The results of the callus formation percentage showed that stem tissue and leaf + node tissue exhibited the highest percentage of callus formation (100%). The shoot tip tissue showed 93% callus formation, and the leaf tissue showed 90%.

Degree of Callus Browning

To evaluate the degree of browning of the calluses, the browning and darkness of the calluses were categorized into five ranks. The lowest amount of browning was ranked as number one, while the highest amount was ranked as number five. The results of the investigation revealed that the tissues of the shoot tip, leaf, and leaf + node did not differ from each other; all three tissues were ranked number one, indicating the least amount of browning. However, the stem

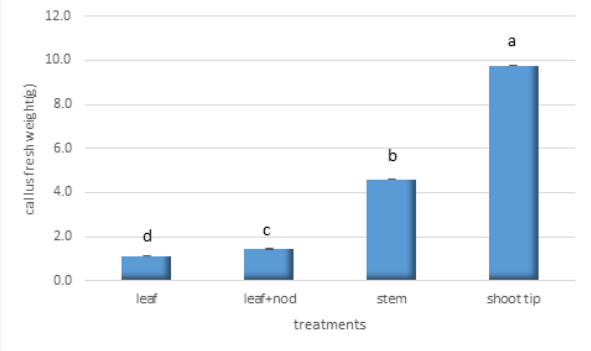

Fig. III. Fresh Weight Analysis of Four *Taxus baccata* Tissue Types

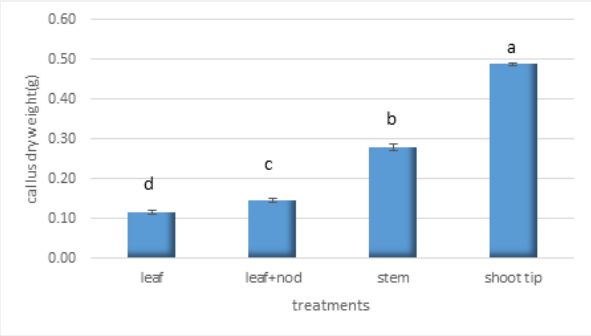

Fig. IV. Dry Weight Analysis of Four *Taxus baccata* Tissue Types

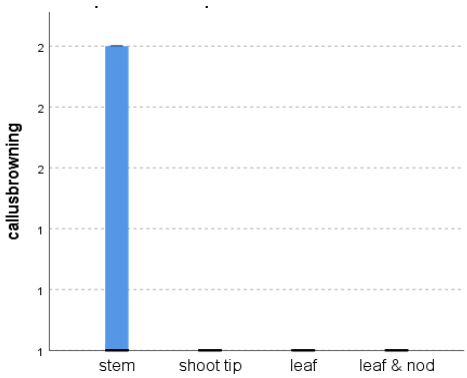

Fig. V. Callus Browning in Four *Taxus baccata* L. Tissue Types

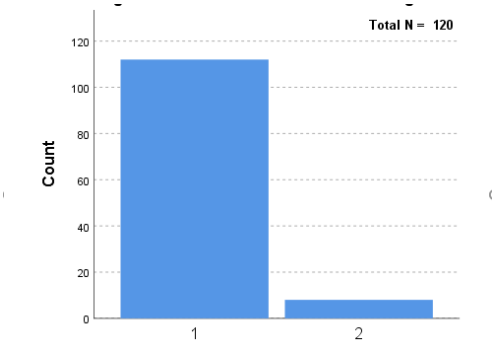

Fig. VI. Count of Callus Browning in Four *Taxus baccata* L. Tissue Types

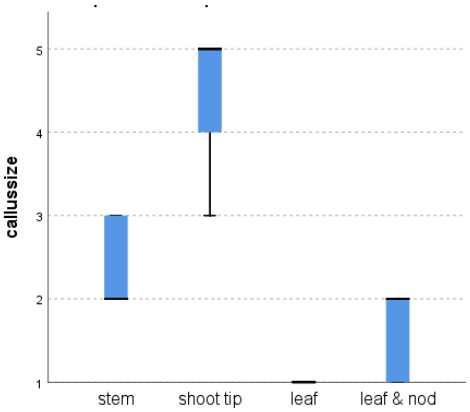

Fig. VII. Callus Size in Four *Taxus baccata* L. Tissue Types

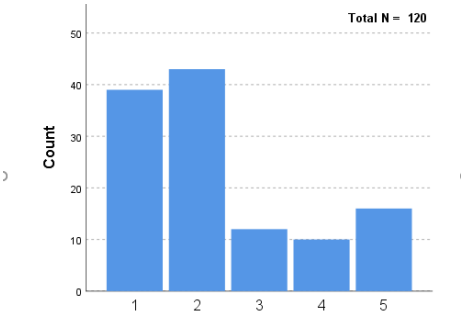

Fig. VIII. Count of Callus Size in Four Different Types of Tissue

tissue differed from the other tissues, with a ranking of number two, indicating that the amount of browning was between low and medium. Among the thirty stem tissue samples, only eight showed a ranking of number two, while the remaining samples were ranked number one, meaning that 26.6% of the stem tissue samples showed slight browning, while the rest exhibited no browning, similar to the other tissues (Fig. V).

Therefore, as shown in Fig. VI, among the total of 120 samples for the four tissue types, the frequency of rank number one was higher than the other ranks. Rank number two was very rare, and the other ranks were not observed. Overall, the results indicate that the amount of browning of the calluses was low, as reflected by the higher frequency of rank number one.

Callus Size

To determine the size of the callus, the callus size was classified into five ranks. The smallest callus size, with a diameter of less than 0.5 cm, was assigned number 1, while the largest callus size, with a diameter greater than 1 cm, was assigned number 5. According to Fig. VII, the largest callus size is related to the tissue of the shoot tip. The shoot tip tissue, with the largest callus size, was assigned number 5, followed by number 4 and a smaller number of number 3. In fact, 60% of the data corresponds to number 5, which represents the largest callus size. After the shoot tip tissue, the stem tissue exhibited the largest callus size, with its data falling between numbers 2 and 3, indicating an average size. The leaf + node tissue followed, with a callus size between numbers 1 and 2. The leaf tissue had the smallest callus size, which was assigned number 1.

According to Fig. VIII, among the 120 samples representing the four tissue types, the most frequent ranks were numbers 1 and 2, which corresponded mainly to the leaf tissue and leaf + node tissue. The frequency of number 5 was higher next, and all of these were related to the data of the shoot tip tissue.

Discussion

Considering the importance of the yew plant due to the presence of the valuable anti-cancer substance Taxol and its extinction, tissue culture of this vital plant is crucial, and research using modern biotechnology methods on this plant is essential(Bangash et al.). In *Taxus baccata* explants, controlling pollution and inhibiting browning are some of the challenges in tissue culture(Hao, 2021). Therefore, necessary measures to solve these problems are vital in research related to the tissue culture of this valuable species (Tomilova et al., 2023). In this research, sterilization protocols for explants were used according to the sources. Due to the presence of a small percentage of contamination, newly grown branches were used as explants, as they had less contamination, and as a result, no contamination was observed.

The results showed that the percentage of callus formation was highest in the stem and leaf + node tissue. The fresh and dry weight results indicated that the shoot tip tissue produced the most callus. Therefore, the type of plant explant can significantly affect both the formation and the quantitative and qualitative parameters of the callus(Ghasemnezhad et al., 2024). Selecting the explant at the optimal growth stage can play a key role in the success of in vitro tissue culture (Mehbub et al., 2022). The high amount of callus weight in the shoot tip tissue is most likely due to the accumulation of plant growth regulators in the shoot tip, which aid in the formation of more callus(Martinez et al., 2021). The amount and ratio of plant growth regulators are crucial factors in callus formation(Guo and Jeong, 2021).

The results of the callus browning showed that the stem tissue browned more than the other tissues, which is likely due to the accumulation of more phenolic compounds in the stem tissue. In most woody plants, as well as some grass species grown in vitro, the explants turn brown and produce phenolic compounds (Rybin et al., 2024). Browning of explants and the loss of explants during the first stage of plant tissue culture is a frequent problem(Devi et al., 2024). One of the most significant issues in callus formation, especially in medicinal plants, is callus browning, which leads to decreased cell growth or the cessation of cell growth, reduced regeneration potential, and, ultimately, cell death (Liu et al., 2024).

In most plant tissue culture methods, explants are first cut and then placed into culture medium, which can create stressful conditions. This can result in the production of phenolic compounds in the explants or the culture medium (Taghizadeh et al., 2024). In research on yew callus cultivation, the most common problem is the browning of the callus tissue and the slow growth of yew callus due to the presence of phenolic compounds (Yao et al., 2023). Neutralizing or removing phenols and preventing browning of plant tissues are essential factors in plant tissue culture research that impact the success of these projects. To prevent tissue browning, various methods have been proposed, such as keeping calluses in the dark or using antioxidants that prevent oxidative stress and the oxidation of phenolic compounds (Liu et al., 2024).

Additionally, the use of activated charcoal and polyvinyl pyrrolidone (PVP), which reduce the toxicity of these compounds, can be mentioned (Permadi et al., 2024).

Conclusion

In this research, the results of examining and comparing four different types of *Taxus baccata* L. tissue in terms of morphological traits and callus induction showed that the tissue of the shoot tip has the lowest amount of browning and the highest amount of callus production and callus size. Therefore, to succeed in yew callus cultivation and to better advance many research in yew tissue culture and reduce the risk of extinction of this valuable species, the shoot tip tissue can be used as an explant.

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