DOI : 10.58993/ijh/2024.81.3.1

Indian J. Hortic. 81(3), September 2024: 223-230

Callus induction, growth, and metabolite variations in two *Taxus* spp. under *in vitro* conditions

Arezoo Jondoaghleboob, Azim Ghasemnezhad*, Mostafa Khoshhal Sarmast and Kamran Rahnama**

Department of Horticultural Sciences, Gorgan University of Agricultural Sciences and Natural Resources, 49138-15739, Iran

ABSTRACT

The present study investigated the effect of growth regulators on the quantity and quality of calli of two yew genotypes (*Taxus* spp.). A factorial experiment based on a completely randomized design was performed using 2,4-D and kinetin on leaf and stem explants of *T. baccata* and *T. brevifolia*. Calli traits, including fresh weight, total phenols, total alkaloids, and paclitaxel contents were investigated. The simple effect of hormonal treatments on total phenolic content, total alkaloids, and fresh weight of calli was significant. Total phenols content and fresh weight were not much affected by the interaction of hormonal treatments, whereas total alkaloids content was found increased. Paclitaxel content did not significantly differ between explants. The highest paclitaxel content was found in the leaf explant of *T. baccata* (30 µg/g), compared to 5 and 10 µg/g in *T. baccata* stem, *T. brevifolia* stem, and *T. brevifolia* leaf, respectively. The fresh weight and total alkaloid content of stem calli of both species were higher than the leaves. Yew is a valuable endangered medicinal plant that responds well to *in vitro* treatments. Therefore, it is possible to manage the production of its valuable metabolites under *in vitro* conditions.

Key words: Alkaloids, metabolites, paclitaxel, taxol, tissue culture.

INTRODUCTION

Plants have been a crucial source of medicine since ancient times, and their importance in modern medicine continues to grow. The World Health Organization estimates that over 80% of people rely on plants in traditional and/or modern medicine, with many synthetic drugs derived from plant-based chemicals (Tripathi and Tripathi, 18). The yew tree (*Taxus* spp.), a rare and endangered conifer species found in the ancient Hircanian forests of northern Iran (Alavi *et al.*, 2), is particularly valuable for producing paclitaxel—a compound approved by the USFDA in 1997 for treating uterine and breast cancer. However, paclitaxel extraction is resource-intensive; producing one kilogram requires ten tons of yew bark and wood, leading to the felling of approximately eight 60-year-old trees for a single patient's treatment, further endangering the species (Sonia *et al.*, 16).

Given these challenges, callus cultures of *Taxus* species have emerged as a promising alternative for paclitaxel production. These cultures have been shown to produce significant levels of paclitaxel, offering a sustainable and cost-effective method to meet demand. Additionally, callus cultures are rich in phenolic compounds, including flavonoids and tannins, which have been linked to paclitaxel biosynthesis and possess notable antioxidant and antimicrobial properties.

This study aims to evaluate the yield and components of paclitaxel in *T. baccata* and *T. brevifolia* under different media conditions and explant types. By exploring alternative methods for paclitaxel production, this research seeks to protect the endangered species like the yew tree and ensure the sustainable production of this vital anticancer drug.

MATERIALS AND METHODS

The present study was conducted in the Tissue Culture Laboratory of the Department of Horticultural Sciences, Gorgan University of Agricultural Sciences and Natural Resources. The young, non-polluting stems of *Taxus baccata* and *T. brevifolia* were collected from Botany Garden of University and the National Botanic Garden of Noshahr, respectively.

Around 5 cm terminal section of suitable stems were separated from the original sample and rinsed in water containing a few drops of dishwashing liquid for 10 minutes. In order to completely remove the residue, several washes were performed in running water and the samples were immediately transferred under a laminar flow hood. The plant materials were treated with 70% alcohol for 15 s. Immediately, the samples were disinfected with sodium hypochlorite

*Corresponding author: Ghasemnezhad@gau.ac.ir

**Department of Phytopathology, Gorgan University of Agriculture and Natural Resources, Iran

(NaOCl) containing 5% activated chlorine and a drop of dishwashing liquid for 25 min. Finally, one-centimeter-long explants were prepared from disinfected plant organs. Explant were cultured on the B5 medium containing 6 levels of a combination of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin including concentrations of 1, 2 and 3 mg/l 2,4-D and 0.2 and 0.5 mg/l kinetin in 3 replications. The cultures were kept in growth room with the temperature and day light of 24 to 26°C and 16:8 h of light and dark conditions, respectively. Four weeks after culture, sub-culture was performed. The resulting callus samples were then used for measuring morphological traits such as colour intensity, tissue thickness, and growth rate and biochemical characters such as total phenols, total alkaloids and the amount of paclitaxel.

The total phenolics content of the calli was measured using the Slinkard *et al.* (17) method. Total alkaloids were extracted from three grams of callus using a modified method by Zarezadeh *et al.* (19). For paclitaxel extraction, the method of Ghasempoor *et al.* (10) was followed. The methanolic extract was rotary evaporated to remove dichloromethane, dissolved in 5 ml of high-purity acetonitrile, and stored at -20°C until analysis. The paclitaxel content, reported as mg/g of fresh weight, can be compared with other studies to identify callus lines with higher yields for further investigation.

The amount of paclitaxel in the stem and leaf of *T. baccata* and *T. brevifolia*, as well as in the callus derived from these species, was determined using high-performance liquid chromatography (HPLC) with a Merck-Hitachi system. A C68 column (4.6 × 250 mm) was used, with a mobile phase of methanol, acetonitrile, and water (40:40:20, v/v/v) at a flow rate of 1 mL/min. UV detection was set at 230 nm, with an injection volume of 20 µL for both standards and samples (Fig. 1a and b). Samples were filtered through a 0.42 µm filter before injection. Paclitaxel content was calculated using a Paclitaxel-USA standard and reported as mg/g fresh weight of callus. HPLC's precision allows for accurate quantification of paclitaxel, providing insight into the potential of these callus cultures as a sustainable and cost-effective source of this valuable drug.

The study employed a completely randomized design with a factorial arrangement of 6 treatments and 3 replications. Treatments included three levels of 2,4-D (1, 2, and 3 mg/L) and two levels of kinetin (0.2 and 0.5 mg/L), applied to two species (*T. baccata* and *T. brevifolia*) and two organ types (stems and leaves). Data were analyzed using SPSS version 16, and mean values were compared using the least significant difference (LSD) test at a 5% probability level. Graphs were created with Microsoft Excel 2010. This factorial design allows for the investigation of multiple factors and their interactions on callus production, optimizing growth regulator conditions. Statistical analysis and LSD testing identify significant differences between treatments, while graphing aids in visualizing results.

RESULTS AND DISCUSSION

Surface microbial contamination is a major challenge in *in vitro* culture of woody perennials, especially when explants are collected from the wild. Effective surface sterilization is crucial to prevent fungal and bacterial contamination. Contaminants can originate from microorganisms on the surface, in gaps, or on emerging leaves and buds. Proper disinfection of explants is essential for successful tissue culture (Sarmast, 15). In this study, various surface disinfection methods were tested due to high contamination levels. For stem samples, the optimal method was 15 s of 70% ethanol followed by 25 min of NaOCl (5% active chlorine). For leaves, 5 s of 70% ethanol and 20 min of NaOCl provided the best results (Table 1). Increasing ethanol exposure temporarily reduced contamination

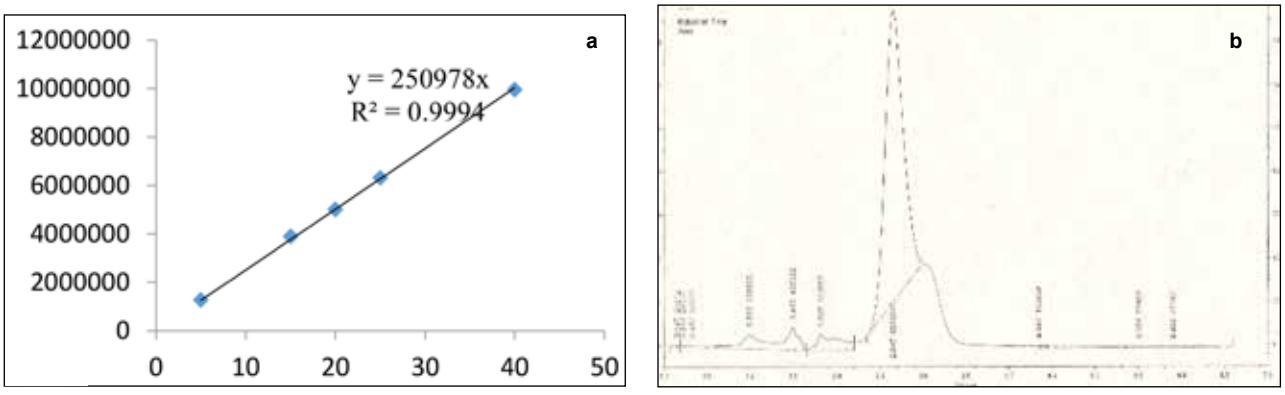

Fig. 1. Calibration curve (a) and Chromatogram (b) of Paclitaxel.

but also decreased explant viability. Ethanol helps the disinfectant penetrate the tissue by removing the cuticle layer but should be used briefly to avoid toxicity (Assareh *et al.*, 3). Previous studies, such as those on *Juniperus communis*, have shown that short ethanol exposure combined with NaOCl effectively controls fungal contamination (Sarmast, 15).

Callus formation from stem explants in both *T. baccata* and *T. brevifolia* began 15 days after culture initiation, while *T. baccata* leaves formed callus in just 12 days. Callus formation in *T. brevifolia* leaves occurred slightly later than in *T. baccata* (Fig. 2). In *T. baccata*, callus developed from the leaf explant, whereas in *T. brevifolia*, it was confined to the base of the petiole. Ashrafi *et al.* (4) reported better cell proliferation in stem explants due to the larger surface area for absorption, suggesting that callus formation from cambium and outer parenchymal tissues is more effective.

Leaf explants generally produced smaller and weaker callus compared to stem explants, aligning with previous findings that stem explants are more effective for callus formation (Ashrafi *et al.*, 4). Among the treatments, stem explant showed the highest callus growth rate, followed by petiole and leaf (Fig. 2). Initially, the callus was green, but to prevent discolouration and promote growth, subculturing was done after 4 weeks with medium containing 200 mg/L activated charcoal. The calli turned orange-brown and maintained a soft, friable texture. The quality of callus is influenced by the culture medium and growth regulators. Media with low kinetin and high 2,4-D concentrations promote rapid callus growth with a soft, loose structure (Davarpanah *et al.*, 8), which was observed in this study. *T. baccata* showed greater callus formation potential than *T. brevifolia*, possibly due to differences in environmental and geographical conditions. Variations in callus production across species and subspecies are influenced by culture media and hormonal composition (Brunakova *et al.*, 6).

Fig. 2. Callus formation in *T. baccata* and *T. brevifolia* stem and leaf explants. (1) *T. baccata* stem, (2) *T. baccata* leaf, (3) *T. brevifolia* stem, and (4) *T. brevifolia* leaf.

Table 1. Effect of surface sterilization on microbial contamination and necrosis of stem and leaf explants of two yew species under *in vitro* conditions.

Plant species	Treatment details	Microbial contamination (%)	Necrosis (%)
Taxus baccata			
Stem	30 s ethanol 70% + 20 m sodium hypochlorite 5%	35.5	86.1
	50 s ethanol 70% + 25 m sodium hypochlorite 5%	62	5.5
	15 s ethanol 70% + 25 m sodium hypochlorite 5%	4.7	0
Leaf	20 s ethanol 70% + 10 m sodium hypochlorite 5%	0	100
	20 s ethanol 70% + 15 m sodium hypochlorite 5%	60	61.1
	5 s ethanol 70% + 20 m sodium hypochlorite 5%	10	0
Taxus brevifolia			
Stem	30 s ethanol 70% + 20 m sodium hypochlorite 5%	0	100
	50 s ethanol 70% + 25 m sodium hypochlorite 5%	66.6	11.1
	15 s ethanol 70% + 25 m sodium hypochlorite 5%	11.6	11.1
Leaf	20 s ethanol 70% + 10 m sodium hypochlorite 5%	0	100
	20 s ethanol 70% + 15 m sodium hypochlorite 5%	36.11	100
	5 s ethanol 70% + 20 m sodium hypochlorite 5%	0	44.4

This study examined the effects of 2,4-D and kinetin on callus formation in stem and leaf explants of *T. baccata* and *T. brevifolia*. Callus formation is essential for plant tissue culture, enabling mass propagation, secondary metabolite production, and studies of plant cell growth and differentiation. Fresh weight of callus after four weeks on Murashige and Skoog (MS) medium with various 2,4-D and kinetin concentrations was used as a measure of callus formation. The results indicated that 2,4-D and kinetin combinations significantly affected callus fresh weight. For *T. baccata* stem explants, the optimal combination was 2 mg/L 2,4-D and 0.1 mg/L kinetin, while for *T. brevifolia* leaf explants, it was 1 mg/L 2,4-D and 0.1 mg/L kinetin. These findings highlight the potential of optimizing growth regulator concentrations to enhance callus formation, which could benefit propagation and secondary metabolite production in these species.

Analysis of variance indicated that both 2,4-D and kinetin, as well as their interaction, significantly affected callus fresh weight (Table 2). Increasing concentrations of both hormones individually led to greater callus fresh weight (Fig. 3 & 4). The interaction of treatments also had a significant effect (Fig. 5). While increasing kinetin concentration did not significantly enhance callus growth compared to 2,4-D, the combinations of 3 mg/L 2,4-D with 0.5 mg/L kinetin and 2 mg/L 2,4-D with 0.5 mg/L kinetin did not show significant differences (Fig. 5). The control treatment, lacking hormonal compounds, resulted in no callus formation, underscoring the necessity of growth regulators for callogenesis (Bagheri and Saffari, 5). For optimal callus production, 3 mg/L 2,4-D was recommended for both stem and leaf explants of *T. baccata*. Consistent with Karimian *et al.* (12), 2,4-D was found to be more effective than Kinetin for *T. brevifolia* stem explants. Mean comparison results (Fig. 6) revealed that the fresh callus weight was lower in *T. baccata* stem and leaf explants compared to *T. brevifolia* stem explants, with *T. baccata* leaf callus formation being less than that from stem explants. These findings align with Razavi *et al.* (14), who reported higher callus

Table 2. Analysis of variance of the effect of 2,4-D, kinetin and their interaction on total phenols, total alkaloids and total fresh weight of callus.

Source of variation	df	Total phenols (µmf)	Total alkaloids	Total fresh weight	Total sugars (µmf)
2,4-D	3	50.119**	13.74**	12.318**	2.845^{ns}
Kinetin	2	0.001^{ns}	13.74**	36.739*	0.319^{ns}
Species	1	58.75**	8.15**	20.119**	32.86**
2,4-D × kinetin	6	8.261**	9.275**	3.996*	0.102^{ns}
Kinetin × Species	2	164.905**	26.494**	24.307**	94.293**
2,4-D × Species	3	4.142**	8.13**	5.685**	0.168 ^{ns}
Species × 2,4-D × kinetin	6	4.078**	3.289*	0.991^{ns}	1.047^{ns}
Error	42	0.001	0.003	0.058	0.002

ns, *, ** : non-significance and significance at the level of 5 and 1%, respectively.

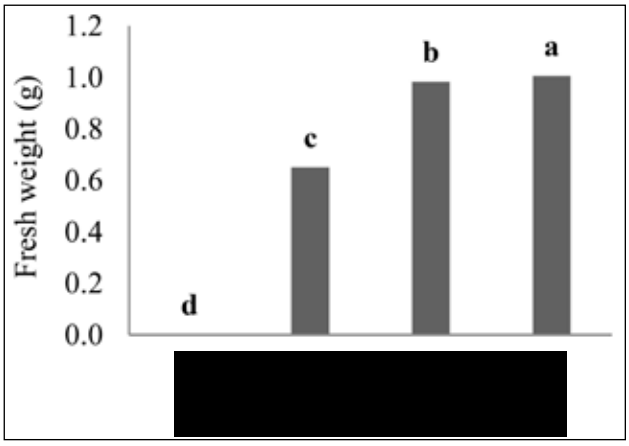

Fig. 3. Effect of 2,4-D on yew callus fresh weight.

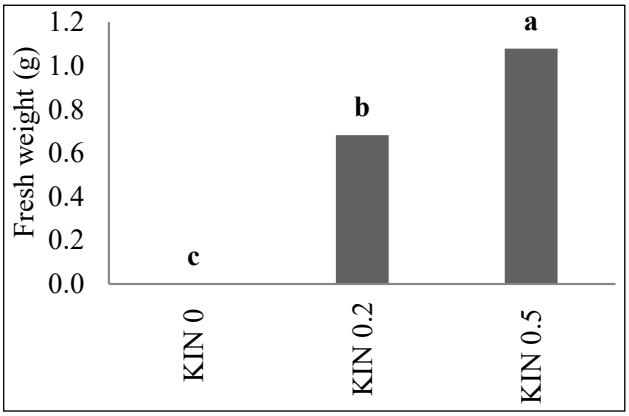

Fig. 4. Effect of kinetin on yew callus fresh weight.

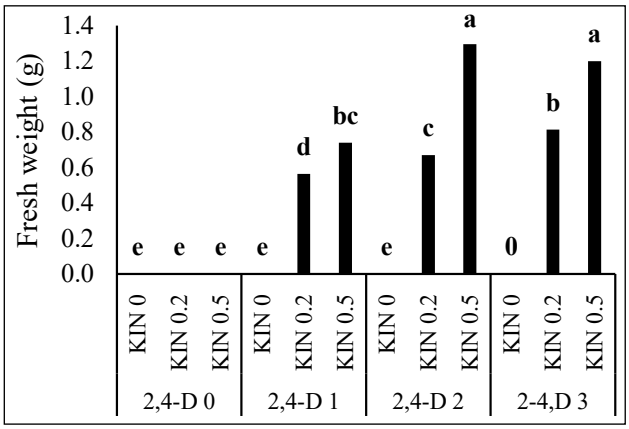

Fig. 5. Interaction effect of treatments on callus fresh weight.

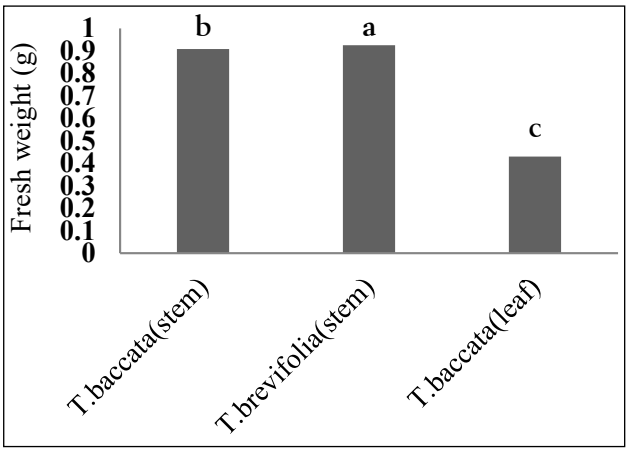

Fig. 6. Callus fresh weight included of different yew species.

formation in *T. baccata* stem explants compared to leaf explants.

This study investigated the effects of various levels of 2,4-D and kinetin on the total phenols content in callus cultures of *T. baccata* and *T. brevifolia*. Results showed significant effects from 2,4-D, the interaction of 2,4-D and kinetin, and the combination of 2,4-D, kinetin, and species on total phenols content (Table 2). Increasing 2,4-D concentrations led to decreased phenol levels (Fig. 7), with leaf explants producing less phenol than stem explants, although no significant difference was found between stem explants (Fig. 8). Phenol content decreased with higher 2,4-D and kinetin concentrations (Fig. 9), and non-significant differences were observed between certain treatment combinations. Appropriate concentrations of 2,4-D enhanced phenolic compounds production in yew callus cultures, which is important for producing bioactive compounds. However, browning, caused by factors such as explant necrosis, pathogen contamination, phenol oxidation, and high sterilizer concentrations, remains a challenge and can affect

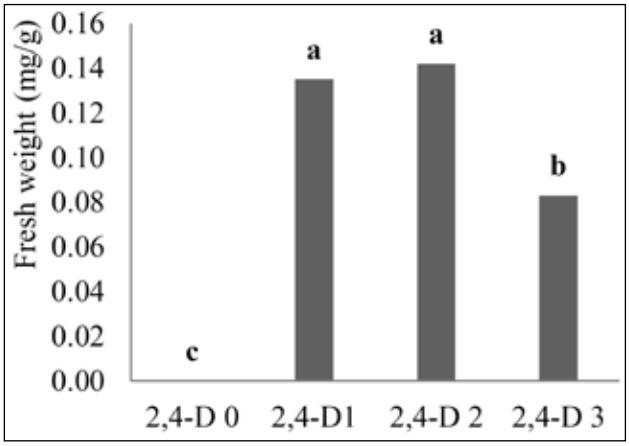

Fig. 7. Effect of 2,4-D on total phenols content of callus.

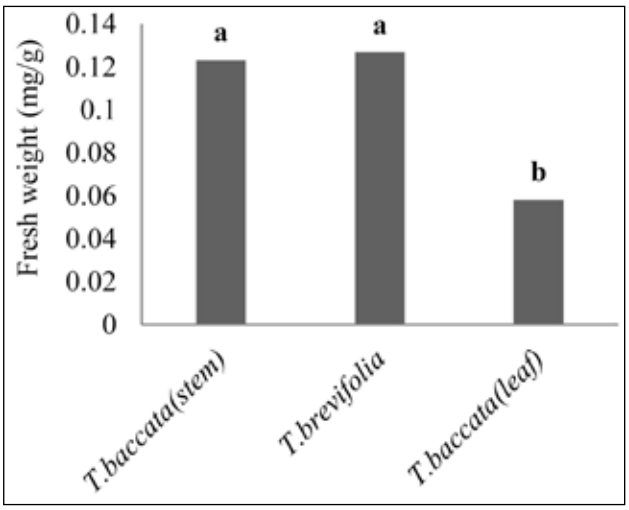

Fig. 8. Effect of plant species on total phenols content of callus.

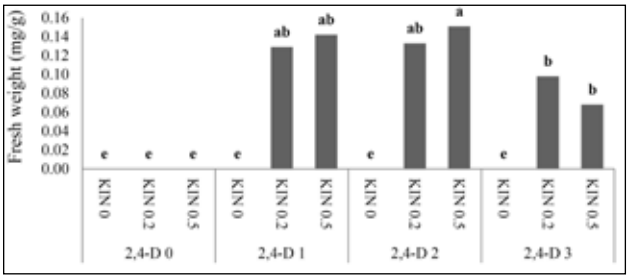

Fig. 9. Interaction effect of 2,4-D and kinetin on the total phenols content of yew callus.

phenolic compound production (Chee, 7). Therefore, strategies to minimize browning are needed. Overall, manipulating plant growth regulators significantly impacts phenolic compound production in yew callus cultures, warranting further research into the specific

phenolic compounds and their pharmacological properties.

Analysis of variance (Table 2) revealed that total alkaloid content in *T. baccata* and *T. brevifolia* was significantly affected by both 2,4-D and kinetin, whether used alone or in combination. Total alkaloids accumulation increased with higher levels of both growth regulators, with stem explants showing higher alkaloid levels compared to leaf explants (Fig. 10). The interaction of 2,4-D and kinetin (Fig. 11) demonstrated that increasing kinetin concentration from 0.2 to 0.5 mg/L enhanced alkaloid levels, irrespective of 2,4-D concentration. Kinetin appeared to be more effective than 2,4-D in increasing alkaloid content. Unlike phenolic compounds, alkaloid levels did not significantly change with increasing 2,4-D concentrations. These results indicate that while both 2,4-D and kinetin can enhance alkaloid production, Kinetin may have a more pronounced effect.

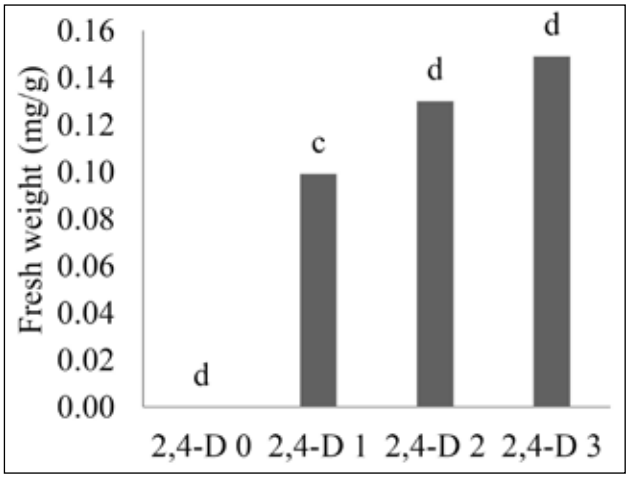

Fig. 10. Effect of 2,4-D on the total alkaloids content.

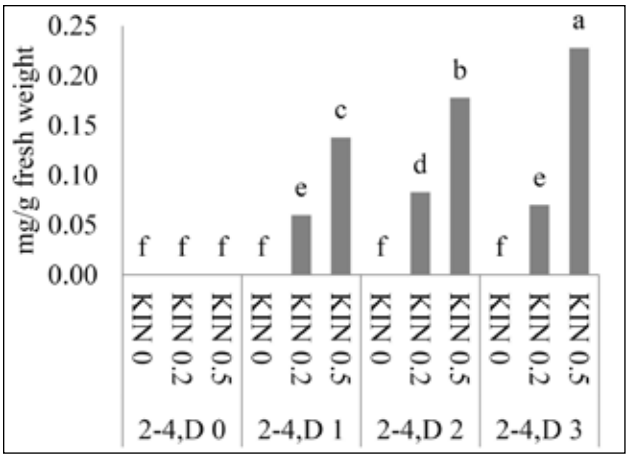

Fig. 11. Interaction effect of kinetin × 2,4-D on the total alkaloids.

Fig. 12 shows significant differences in total alkaloid content between calli from *T. baccata* leaf and stem explants compared to calli from *T. brevifolia* stem explants under the combined influence of 2,4-D, kinetin and species. No significant difference was found between *T. baccata* and *T. brevifolia* in medium with 2 mg/L 2,4-D and 0.5 mg/L kinetin, though differences were noted within *T. baccata* explants. These results suggest that genetic differences between species affect alkaloid production in callus cultures, highlighting the need for species-specific optimization of growth conditions and regulators. Khataee and Karimi (13) found that combinations of BA (benzyladenine) and NAA (naphthalene acetic acid) significantly increased alkaloid content in callus, indicating that appropriate plant growth regulator combinations can enhance alkaloid production. Further research is needed to identify specific alkaloids and their pharmacological potential.

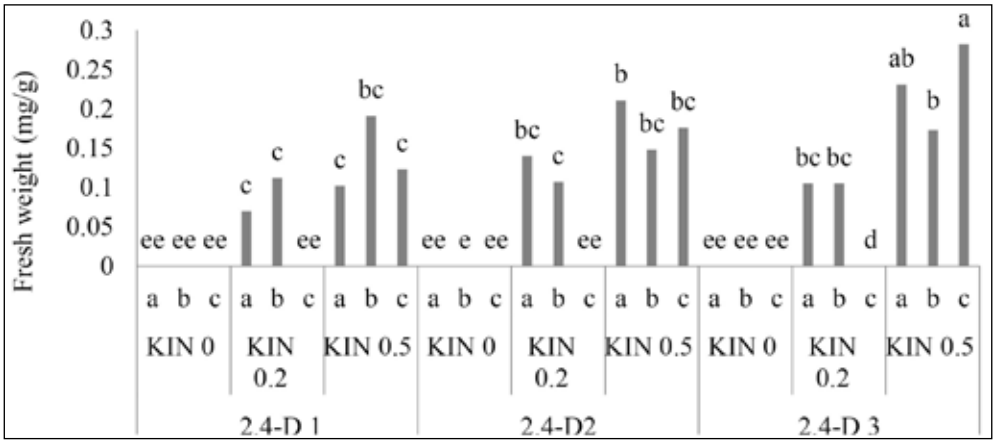

Fig. 12. Total alkaloids content affected by complex effect of treatments. a = stem callus of *T. baccata*. b = leaf callus of *T. baccata*. c = stem callus of *T. brevifoli.*

The production of tropane alkaloids in tissue culture is highly influenced by the composition of the culture medium, including nutrient sources, growth regulators, and growth conditions (Iranbakhsh *et al.*, 11). Optimal production requires careful selection and adjustment of these factors. Appropriate combinations of growth regulators, such as auxins and cytokinins, have been shown to enhance alkaloid production. Additionally, elicitors like methyl jasmonate and salicylic acid can stimulate alkaloid production (Ebrahimi *et al.*, 9). Understanding these factors is crucial for developing efficient methods for producing tropane alkaloids and optimizing their pharmacological properties. Further research is needed to explore the mechanisms regulating alkaloid biosynthesis in tissue culture.

The results of this study showed that the amount of paclitaxel in *T. baccata* stem callus was significantly higher (33.36 µg/g) than that of *T. brevifolia* stems (26.45 µg/g) and leaves (23.28 µg/g). However, no significant difference was observed between the paclitaxel content of calli derived from *T. brevifolia* stem and leaf explants. Despite the fact that paclitaxel was first identified in *T. brevifolia*, studies have shown that the amount of this compound and its derivatives in some other species of this genus, such as *T. baccata*, is higher than that of *T. brevifolia* (Fig. 13). However, it should be noted that the rate of paclitaxel varies from species to species and even from organ to organ, and can vary between 0 and 500 µg/g dry weight. Previous studies by other researchers (Ahadi *et al.*, 1) have reported that, in general and under natural conditions, the potential of paclitaxel production is higher in *T. baccata* compared to *T. brevifolia*. This tendency was also observed in the *in vitro* studies of the present study. These findings suggest that tissue culture techniques can be used to produce paclitaxel and other important metabolites in a controlled and sustainable manner. Further studies are needed to optimize the production of specific metabolites with potential pharmacological properties and to elucidate the underlying mechanisms of their biosynthesis in tissue culture.

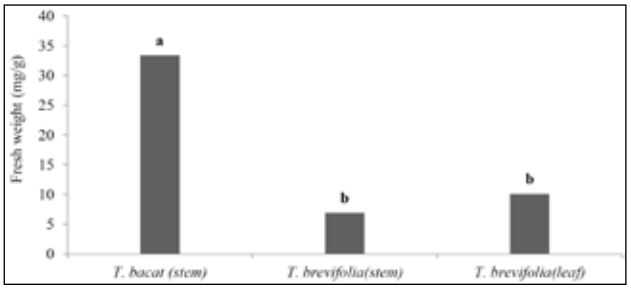

Fig. 13. Mean value of paclitaxel affected by plant species.

The study highlights that effective tissue culture disinfection methods must be tailored to the specific plant species and organs used. For callus induction in yew trees, the optimal hormonal treatment was 3 mg/L 2,4-D combined with 0.2 mg/L kinetin. Under these conditions, *T. brevifolia* callus had a higher fresh weight than *T. baccata*, though *T. baccata* stem callus was heavier than its leaf callus. Increasing 2,4-D and kinetin concentrations enhanced total alkaloid content, with stem-derived callus having higher alkaloid levels compared to leaves. Conversely, higher 2,4-D concentrations reduced the total phenols content, with leaf explants oozing less phenols than stems. No significant difference in stem phenolization rates between taxa species was observed. Future studies should explore varying hormone concentrations and metabolic conditions in the culture medium to optimize the metabolite production with potential pharmacological benefits.

AUTHORS' CONTRIBUTION

Conceptualization of research (AG); Designing of the experiments (AJ and MS); Contribution of experimental materials, Preparation of the manuscript (KR).

DECLARATION

The authors declare that there is no conflict of interest.

ACKNOWLEDGMENTS

The authors thank the Vice Chancellor for Research of Gorgan University of Agricultural Sciences and Natural Resources for providing research facilities.

REFERENCES

1. Ahadi, H., Mirjalili, M.H., Farzaneh, M., Shirshekan, M. and Ghassempour, A. 2013. Quantification of taxol in wild mature and *in vitro* cell suspension cultures of *Taxus baccata* and *Taxus brevifolia*: A comparatives study. 2nd National Congress on Medicinal Plants, Tehran, 1409 p.

2. Alavi, S.J., Veiskarami, R., Esmailzadeh, O. and Gadow, K. 2020. Analyzing the biological and structural diversity of hyrcanian forests dominated by *Taxus baccata* L. *Forest* **11**: 701-18.

3. Assareh, M.H., Ghorbanli, M., Akbari, M., Ghamari Zare, A. and Emam, M. 2006. Micropropagation, organogenesis and using new method of semi-photoautotrophic in

Eucalyptus gongylocarpa. *Pajohesh-va-Sazandegi* **75**: 134-45.

4. Ashrafi, S., Mofid, M.R., Otroshi, M., Ebrahimi, M. and Khosroshahli., M. 2010. Effect of plant growth regulators on the callogenesis and taxol production in cell suspension of *Taxus baccata* L. *Trakia J. Sci.* **8**: 36-43.

5. Bagheri, A. and Saffari, M. 2011. *In vitro* culture of higher plants, Ferdowsi University of Mashhad Press, 406 p. (In Persian).

6. Bruňáková, K., Babincova, Z. and Cellarova, E. 2004. Selection of callus cultures of *Taxus baccata* L. as a potential source of paclitaxel production. *Eng. Life Sci.* **4**: 465-69.

7. Chee, P.P. 1995. Organogenesis in *Taxus brevifolia* Nutt. tissue cultures. *Plant Cell Rep.* **14**: 560-65.

8. Davarpanah, S.J., Lahouti, M. and Karimian, R. 2015. Study of callus initiation and growth criteria at different concentrations of 2,4-D and kinetin in *Taxus baccata* L. embryo culture. *Iran. J. Plant Biol.* **7**: 41-50.

9. Ebrahimi, M. and Mokhtari, A. 2017. Engineering of secondary metabolites in tissue and cell culture of medicinal plants: an alternative to produce beneficial compounds using bioreactor technologies. **In***: Crop improvement.* Abdullah, S., Chai-Ling, H. and Wagstaf, C. (Eds.), Springer, Cham, pp. 137-67.

10. Ghassempour, A., Rezadoost, H., Ahmadi, M. and Aboul-Enein, H.Y. 2009. Seasons study of four important taxanes and purification of 10-deacetylbaccatin III from the needles of Taxus baccata L. by two-dimensional liquid chromatography. *J. Liq. Chromatogr. Relat. Technol.* **32**: 1434-47.

11. Iranbakhsh, A., Oshaghi, M. and Ebadi, M. 2007. Growth and production of tropane alkaloids in *Datura stramonium* cell suspension culture. *Pak. J. Biol. Sci.* **10**: 1236-42.

12. Karimian, R., Lahouti, M. and Davarpanah, S.J. 2014. Effect of different concentration of 2,4-D and kinetin on callogenesis of *Taxus brevifolia* Nutt. *J. Appl. Biotechnol. Rep.* **1**: 167-70.

13. Khataee, E. and Karimi, F. 2010. Auxin and cytokinin effects on callus and organ production and total alkaloid contents in *Datura innoxia* calli. *Iran. J. Plant Biol*. **2**: 55-66.

14. Razavi, S.A., Hosseini Nasr, S.M., Reza Doost, H. and Rostami Charati, F. 2017. Effects of IBA, NAA and 2,4-D on callus production and growth in common yew (*Taxus baccata* L.) under *in vitro* conditions. *J. Wood For. Sci. Technol.* **24**: 1-16.

15. Sarmast M.K. 2018. *In vitro* establishment of conifers by mature shoots. *J. For. Res.* **29**: 565-74.

16. Sonia, M., Rosa, M., Mirjalili, M.H., Moyano, E., Javier, P. and Bonfill, M. 2011. Production of the anticancer drug taxol in *Taxus baccata* L. suspension culture: A review. *Process Biochem.* **46**: 23-34.

17. Slinkard, K. and Singleton, V.L. 1977. Total phenol analysis: automation and comparison with manual methods. *Amer. J. Enol. Vitic.* **28**: 49-55.

18. Tripathi, L. and Tripathi, J.N. 2003. Role of biotechnology in medicinal plants, *Trop. J. Pharm. Res.* **2**: 243-53.

19. Zarezadeh, A., Khold Barin, B., Moradshahi, A., Babakhanlou, P. and Rajaei, H. 2000. Changes in total alkaloid substances in physalis alkekengi in response to nitrogenous fertilizer. *Iran. J. Med. Aromat. Plants.* **5**: 61-12.

Received : July, 2024; Revised : September, 2024;
Accepted : September, 2024