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# Effect of Plant Growth Regulators on Callus Coloration and Glycosides Accumulation in *Tulipa gesneriana* Tissue Culture

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**Abstract**

The tulip (*Tulipa gesneriana*) is an ornamental herbaceous plant. The aim of this study was to evaluate the effects of plant growth regulators on the evolution of colourful callus in this plant and their relevance to the build-up of certain active glycosides, such as 6-tuliposide A, 6-tuliposide B, tulipalin A, and tulipalin B. The growth regulators 2,4-Dichlorophenoxyacetic acid (2,4-D), Benzyladenine (BA), and Thidiazuron (TDZ) were applied at varying doses in four treatments, and the color, texture, and induction rate of the ensuing callus were assessed. The experiment was conducted using a completely randomized design with ten replicates per treatment under controlled in vitro conditions at the Tissue Culture Laboratory, College of Science for Women, University of Baghdad, Iraq, during the winter season of 2024-2025. The results showed a clear variation in the colors of callus with yellow, green, cream, and red colors recorded. The red callus produced from the (2,4-d one + tdz 1) discourse had the ultimate collection of active compounds recording the highest concentrations of 6-tuliposide A (500.10 µg/ml) and 6-tuliposide B (502.50 µg/ml). In contrast, the cream callus produced from the (2,4-D 1.5+BA 1.5) treatment showed the lowest concentrations of Tulipalin A (81.76 µg/mL) and Tulipalin B (126.43 µg/mL), indicating a close relationship between callus color, the level of metabolic activity, and the synthetic pathways involved in the production of secondary products.

**Keywords:** *Tulipa gesneriana*, Plant growth regulators, Colored callus, Tissue culture, Tuliposide, Tulipalin.

## 1. Introduction

Medicinal plants are vital sources of pharmacologically active compounds utilized in disease treatment, as they produce many essential organic chemicals, known as secondary metabolites[1]. A common flowering plant around the world, especially in Europe, is the tulip (*Tulipa gesneriana* L.), a herbaceous ornamental species belonging to the Liliaceae family[2,3]. Turkey, the Netherlands, and Iran are among the countries that have made it their national flower due to its great commercial worth, which is linked to its varied colors, extended blossoming duration, and pleasant aroma[4,5]. Tulip blossoms have a variety of biological functions[6], such as antipyretic, anticancer, laxative, expectorant, and depurative qualities, because of their biochemical composition[7]. Glucose esters made from hydroxy methylenebutanoic acids make up the majority of Tuliposides, which are important secondary metabolites in *Tulipa gesneriana*[8,9]. The lactonized aglycon of tuliposides, tulipalins, is produced when tuliposides are converted by an enzyme known as tuliposide-converting enzyme (TCE)[10,11]. Among the potent substances that greatly enhance antibacterial action is this one[12-14]. High-performance liquid chromatography (HPLC) is one of the most essential tools used to identify and separate these compounds, as it

provides accuracy in identifying secondary plant compounds[15]. Among the advantages of this technique are speed, efficiency, reduced analysis costs, and increased productivity[16].

With the advancement of pharmaceutical sciences and the availability of modern analytical techniques, and given our dependence on these natural compounds to provide safe, low-cost treatments, the harsh environmental conditions in Iraq make it difficult to cultivate many plant species. Therefore, biotechnology provides the ideal solution using plant tissue culture to produce these compounds in the laboratory[17]. An efficient substitute technique for increasing the synthesis of secondary metabolites is plant tissue culture. One of the most widely used in vitro techniques, callus culture, has demonstrated a high level of effectiveness in generating these substances[18, 19] and to produce effective secondary compounds from medicinal plants, in order to meet the increasing demand for them in the industrial and medical fields without affecting the natural sources of plants[20].

This technique makes use of several classes of plant growth regulators, particularly auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) and cytokinins including benzyladenine (BA) and thidiazuron (TDZ). These are active organic substances that are either produced commercially or found naturally in plants. When added during particular periods of plant life, they are utilized in modest amounts to alter plant growth and development. They can be classified as either growth inhibitors or growth boosters[21]. It also affects many physiological and developmental processes, from cell division to organ formation[22].

In comparison to field-grown plants, this method has several advantages, such as being independent of geographic or environmental factors, allowing for control over both production and quality and shortening the plant life cycle. It also removes the demand for land resources[23]. When subjected to specific stressors, callus tissue produced from different explants may produce more secondary chemicals, according to several studies. These substances get extremely work apart sophisticated and are used as useful resources in a number of disparate sectors[24].

In order to adopt this technique as an alternate source for producing pharmaceutical compounds from *Tulipa gesneriana* the research intends to use tissue culture techniques to induce callus from the plant and assess the impact of varying concentrations of plant growth regulators (24-D BA TDZ) on the color and structure of the callus, with an emphasis on the relationship between color and the composition of secondary metabolites, specifically glycosides.

## 2. Materials and Methods

The experiments for this study were conducted during the winter of 2024–2025 at the tissue culture laboratories of the College of Science for Women at the University of Baghdad.

### 2.1. Sterilization of laboratory

Laboratory equipment, such as forceps, scalpels, and Petri dishes, was sterilized in a heat-resistant autoclave at 121°C and 1.04 kg/cm² for 30 minutes. The instruments were then immersed in 96% ethanol, followed by a quick sterilization, always removing any residual contaminants. They were then left to cool inside a laminar airflow cabinet. The interior of the cabinet was also sterilized by ultraviolet light for 30 minutes, followed by a spray with 70% ethanol to ensure sterile conditions and remove any contaminants[25].

### 2.2. Media preparation

Classic MS medium was prepared by dissolving 30 g/L of sucrose in distilled water. The pH was then adjusted to 5.8 using NaOH or HCl. After adding 8 g/L of agar, the medium was autoclaved for 20 minutes and allowed to cool and solidify before use[26].

### 2.3. Seeds germination

After being rinsed under running water and sanitized for 30 seconds in 70% ethanol, the seeds were submerged in a 4% sodium hypochlorite solution while being constantly shaken to promote germination. The seeds were put in universal tubes with MS media after being rinsed three times with sterile distilled water. These tubes were cultured in a growth room at 25°C ± 2 °C with a 16/8-hour light/dark cycle under carefully controlled conditions. Explants were made from the

leaves (0.5 cm long) of seedlings that were 2-3 weeks old[27].

### 2.4. Callus induction

Leaf explants from germinated tulips were aseptically sectioned into 0.2 cm segments. These segments were then placed on MS medium containing 2,4-D, BA, and TDZ, each at concentrations of 0.5, 1, or 1.5 mg/l. Tulip extracts were prepared from intact plants and treated callus. For each sample, 5 g of fresh leaf tissues (intact plant) or callus (wet weight) were mixed with 50 mL of 70% methanol and extracted under ordinary reflux conditions at 60–80 ºC for 6 hours. After extraction, the samples were allowed to cool, filtered, and the resulting crude extracts were stored at 4ºC for subsequent analysis. Glycosides were qualitatively and quantitatively evaluated in the crude extracts using HPLC analysis[28].

### 2.5. Experimental design and statistical analysis

The experiment was arranged in a completely randomized factorial design. Each treatment consisted of ten replicates. Data were analyzed using SPSS (version 26) to evaluate the effects of plant growth regulators (2,4-D with BA or TDZ) and their interactions on callus induction rate. Mean comparisons were performed using the Least Significant Difference (LSD) test at a probability level of $P \leq 0.05$[29].

### 2.6. HPLC Separation of glycosides

The primary chemical was separated using an FLC (Fast Liquid Chromatographic) column, operating under optimum conditions.

- Column: Phenomenex C-18, 50 × 2.0 mm I.D., 3 µm particle size.
- Water containing 0.1% formic acid (solvent A) and acetonitrile (solvent B) constitutes the mobile phase.
- UV at 280 nm for detection
- Rate of flow: 1.0 ml/min

The following were the sequences of the standard's eluted material. 50 µg/ml was the standard for each (**Table 1**).

**Table 1.** Retention times and peak areas of tuliposide and tulipalin standard solutions (50 µg/mL) under optimized HPLC conditions (Primary data obtained from calibration standards analyzed in the present study).

| Seq | Subjects | Retention time (min) | Area (µvolt) | Concentration |
|---|---|---|---|---|
| 1 | 6-tuliposides A | 2.863 | 327877 | 50 µg/ml |
| 2 | 6-tuliposides B | 3.862 | 327995 | 50 µg/ml |
| 3 | Tulipalin A | 4.647 | 393470 | 50 µg/ml |
| 4 | Tulipalin B | 5.423 | 367857 | 50 µg/ml |

### 2.7. Calculation

Sample concentration (µg/ml)= (sample area/standard area) times standard concentration× dilution factor

### 2.8. Apparatus

A Shimadzu LC-10AV liquid chromatography system with a binary delivery pump model LC-10A was used for the separation, and a UV-Vis 10 A-SPD spectrophotometer was used to track the eluted peaks.

## 3. Results

The experiment began by planting *T. gesneriana* seeds on MS medium, which took approximately three weeks to germinate. After the seedlings grew, the leaves were cut into small pieces (explants) and used as a source for callus formation. These were then replanted on nutrient media enriched with various growth regulators to stimulate the morphogenetic response (**Figure 1**).


**Figure 1.** After 20 days, a *T.gesneriana* seedling was cultivated on MS hormone-free medium.

To statistically evaluate the effect of plant growth regulators and their interactions on callus induction rate, factorial analysis was performed as shown in **Tables 2 and 3**.

**Table 2**: Effect of 2,4-D and BA and its interaction in Induction rate (%)

| **2,4-D (mg/L)** | **BA (mg/L)** | | | | **Mean** |
|---|---|---|---|---|---|
| | **0** | **0.5** | **1.0** | **1.5** | |
| 0 | 0 | 0 | 0 | 10 | 2.50 |
| 0.5 | 0 | 30 | 40 | 30 | 25.00 |
| 1.0 | 10 | 40 | 40 | 50 | 35.00 |
| 1.5 | 10 | 40 | 50 | 70 | 42.50 |
| Mean | 5.00 | 27.50 | 32.50 | 40.00 | --- |
| L.S.D. | 2,4-D: 8.027 * * , BA: 8.027 * ,<br>2,4-D x DA: 12.74 *. | | | | |
| * (P<0.05). | | | | | |

**Table 3**: Effect of 2,4-D and TDZ and its interaction in Induction rate (%)

| **2,4-D (mg/L)** | **TDZ (mg/L)** | | | | **Mean** |
|---|---|---|---|---|---|
| | **0** | **0.5** | **1.0** | **1.5** | |
| 0 | 0 | 0 | 0 | 0 | 0.00 |
| 0.5 | 0 | 30 | 30 | 40 | 25.00 |
| 1.0 | 10 | 50 | 50 | 50 | 40.00 |
| 1.5 | 10 | 50 | 60 | 60 | 45.00 |
| Mean | 5.00 | 32.50 | 35.00 | 37.50 | --- |
| L.S.D. | 2,4-D: 7.625 * , TDZ: 7.625 * ,<br>2,4-D x TDZ: 12.819 *. | | | | |
| * (P<0.05). | | | | | |

The cultured explant showed a clear response to callus formation, and the resulting callus exhibited a striking color variation, including yellow, green, cream, and red (**Figure 2**).
Samples showing the highest callus induction rates, characterized by different colors (yellow, green, cream, and red), were selected and extracting. The color variation indicates differences in metabolic activity and possible variations in secondary metabolite accumulation between treatments **(Table 4).**

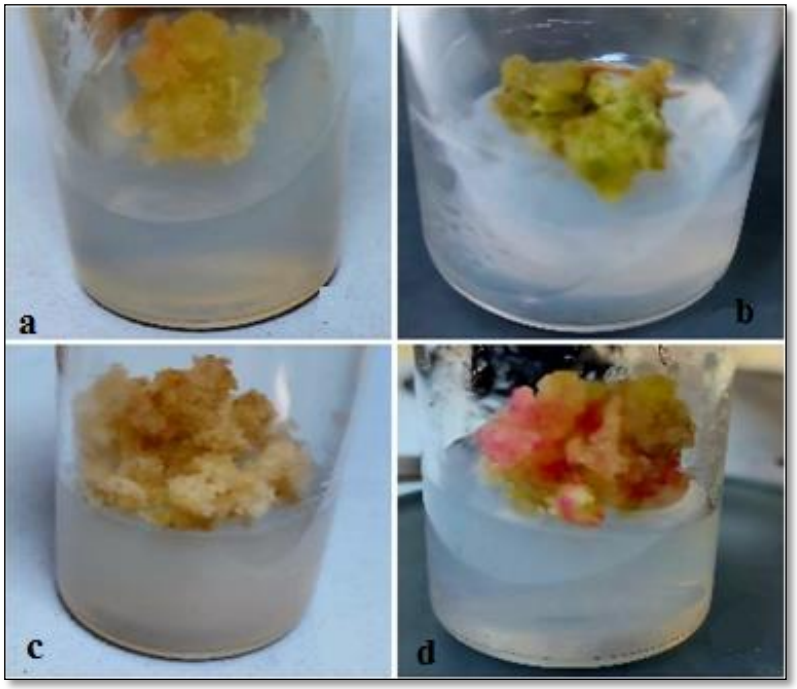


**Figure 2.** Color variation of callus produced from tissue culture of *Tulipa gesneriana* under the influence of different growth regulators. The images show different callus formations with colors ranging from yellow (a), green (b), cream (c), and red (d).

**Table 4.** Impact of several plant growth regulators on *T. gesneriana* callus induction %, texture, and color.

| Plant growth regulators | Callus % | Callus texture | Callus colour |
|---|---|---|---|
| 0.0 | - | - | - |
| 2,4-D (0.5) | 0 | - | - |
| 2,4-D (1) | 10 | friable | yellow |
| 2,4-D (1.5) | 10 | friable | cream |
| 2,4-D (0.5) + BA (0.5) | 30 | friable | green |
| 2,4-D (1) + BA (1) | 40 | friable | green |
| 2,4-D (1.5) + BA (1.5) | 70 | friable | cream |
| 2,4-D (0.5) + TDZ (0.5) | 30 | friable | cream |
| 2,4-D (1) + TDZ (1) | 50 | friable | red |
| 2,4-D (1.5) + TDZ (1.5) | 60 | friable | Yellowish green |

The following callus was selected for study and analysis based on its highest induction rates and color variations:

- Yellow callus with a friable texture that was produced by a 2, 4-D treatment at a dose of 1 mg/L and an induction rate of 10%.
- A 40% induction rate and a friable texture were achieved by the green callus resulting from a treatment with 2,4-D (1 mg/L) and BA (1 mg/L).
- Using 2,4-D and BA at a concentration of 1.5 mg/L for each treatment resulted in a creamy callus with a friable texture and the highest induction rate of 70%.
- Red callus with a friable texture and a 50% induction rate that was produced by a treatment with 2,4-D (1 mg/L) and TDZ (1 mg/L).

Extraction of the active compounds was performed using methanol by the ordinary method. It was observed that the resulting extracts had varying colors, reflecting the chemical content of the callus used (**Figure 3**).

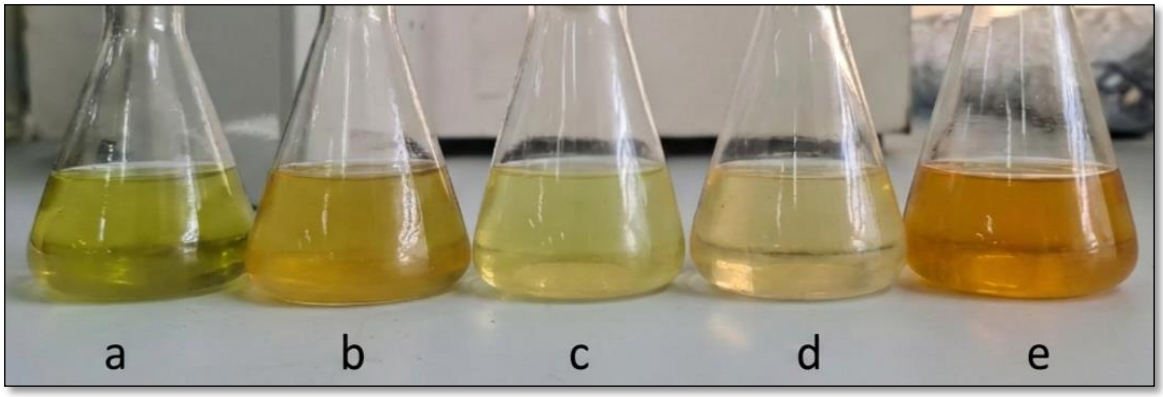


**Figure 3.** Color variation of callus produced from tissue culture of *T. gesneriana* under the influence of different growth regulators. The images show different callus formations with colors corresponding to the following samples: (a) leaf plant extract (dark green), (b) yellow callus, (c) green callus, (d) cream callus, and (e) red callus.

The concentrations of the four compounds (6-Tuliposide A, 6-Tuliposide B, Tulipalin A, and Tulipalin B) varied between treatments when analysed by high-performance liquid chromatography (HPLC). The red callus had the highest concentrations of all the compounds, which may indicate a connection between the rich color of callus and its high secondary compound content (**Figure 4**).

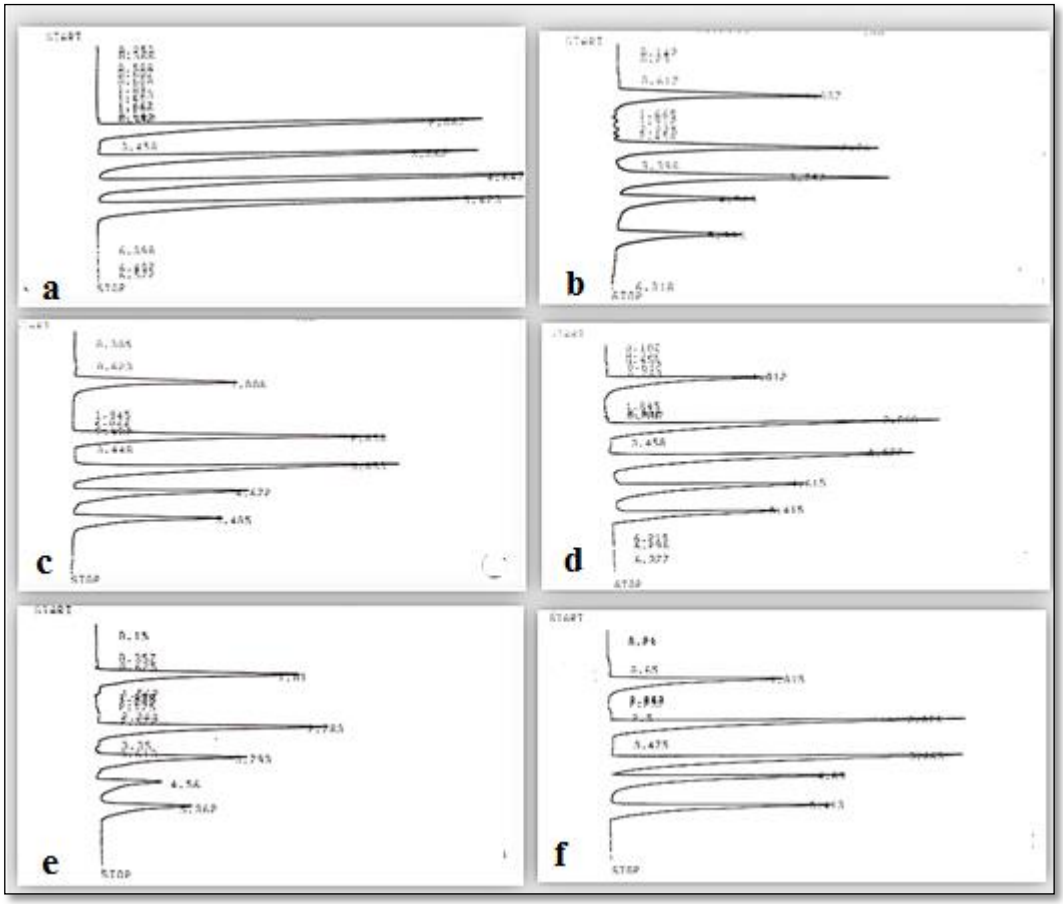


**Figure 4.** HPLC chromatograms of methanolic extracts from different callus colors in *Tulipa gesneriana*: (a) Standard, (b) Control (c) Yellow callus, (d) Green callus, (e) Cream callus, and (f) Red callus.

In comparison to the control sample, **Table 5** displays the amounts of the secondary chemicals 6-Tuliposide A, 6-Tuliposide B, Tulipalin A, and Tulipalin B that were recovered from callus generated by four distinct treatments. In this study, the control sample consisted of extracts

obtained from intact *T. gesneriana* plant tissues, as callus formation did not occur in the absence of plant growth regulator. The results showed a clear variation in the concentrations of the studied compounds between samples, with most compounds recording their highest concentrations in red callus, particularly 6-Tuliposide A (500.10 µg/mL) and 6-Tuliposide B (502.50 µg/mL), indicating the effectiveness of this treatment in stimulating the production of active compounds.

It was also noted that the concentrations of Tulipalin A and Tulipalin B were relatively low in cream callus, reaching (81.76 µg/mL) and (126.43 µg/mL), respectively, while their highest values were recorded in red callus, indicating that the alteration in the color compositions of the callus may be related to changes in the accumulation of these compounds

**Table 5.** Concentration of secondary metabolites (µg/mL) detected by HPLC in different callus samples of *Tulipa gesneriana*.

| Compound | Control | Yellow callus | Green callus | Cream callus | Red callus |
|---|---|---|---|---|---|
| 6-Tuliposide A | 349.18 | 435.63 | 477.40 | 317.12 | 500.10 |
| 6-Tuliposide B | 410.10 | 452.67 | 430.30 | 209.44 | 502.50 |
| Tulipalin A | 179.30 | 208.92 | 264.80 | 81.76 | 284.30 |
| Tulipalin B | 161.50 | 192.05 | 222.70 | 126.43 | 283.50 |

The findings of the HPLC study showed that the percentage of secondary metabolites in the extract was directly impacted by the kind of plant growth regulator employed in callus cultivation (**Table 6**).

The greatest value was found for 6-Tuliposide A (43.2%) in the treatment combining 2,4-D (1.5 mg/L) with BA (1.5 mg/L), which resulted in cream-colored callus and an induction rate of 70%. This suggests that this treatment was the most successful in promoting the synthesis of this chemical.

However, the treatment based on 2,4-D alone (1 mg/L) demonstrated a low percentage of active chemicals and produced yellow callus with a low induction rate (10%), supporting the idea that growth regulators must cooperate to promote advanced chemical synthesis.

6-Tuliposide B was highest in the control sample (36.94%) and dramatically decreased in the 2,4-D + TDZ-treated sample (red callus), which may suggest that TDZ prevented this chemical from accumulating.

Additionally, tulipalin A and B changed from treatment to treatment. Tulipalin B was highest in the 2,4-D + TDZ treatment (red callus), whereas Tulipalin A was highest in the 2,4-D + BA treatment (1 mg/L), which resulted in green callus.

These results suggest that the colour composition of callus may be an indirect indicator of physiological and chemical changes associated with the formation of active compounds.

**Table 6.** Percentages (%) of Tuliposides and Tulipalins in callus extract of *Tulipa gesneriana* under different plant growth regulator.

| Compound | Control | Yellow callus | Green callus | Cream callus | Red callus |
|---|---|---|---|---|---|
| 6-Tuliposide A | 32.35 | 33.79 | 34.2 | 43.2 | 31.8 |
| 6-Tuliposide B | 36.94 | 35.11 | 30.8 | 28.5 | 32 |
| Tulipalin A | 16.15 | 16.2 | 18.9 | 11.12 | 18.1 |
| Tulipalin B | 14.55 | 14.9 | 16 | 17.2 | 18.1 |

## 4. Discussion

The statistical analysis of callus induction rate revealed clear differences among treatments, indicating that the response of *T. gesneriana* explants was strongly influenced by the type and concentration of plant growth regulators. The interaction between auxin (2,4-D) and cytokinins (BA or TDZ) played a key role in regulating cell division and dedifferentiation, leading to variations in callus formation efficiency. Such synergistic effects between auxins and cytokinins have been widely reported in plant tissue culture systems and are attributed to their complementary roles in controlling cellular competence and morphogenic responses.

The data from this study demonstrate that the variation in callus color resulting from the cultivation of plant parts under the influence of different growth regulators reflects notable physiological and chemical changes within the cultured tissue. Callus colors are varied markedly among treatments, exhibiting cream, yellow, green, and red pattern appearance variations in metabolic action and endogenous metabolism, notably in pathways associated with the organisation of secondary metabolites.

With the largest amounts of the four targeted compounds, 6-Tuliposide A, 6-Tuliposide B, Tulipalin A and Tulipalin B, the red callus stood out the most. Increased action in synthesis pathways, including the phenylpropanoid and shikimic pathways, which are important for the synthetic of glycosides and phytochemicals connected to plant defensive responses, may be the cause of this renowned rise.

Alternatively, cream callus, characterized by its pale color and lack of coloration, showed lower concentrations of the above compounds, which may indicate reduced metabolic activity or a deficiency in the gene expression of enzymes responsible for producing the active compounds. This might work because the increase regulators used in this treatment have a poor effect or because the environment is not conducive to encouraging the compounds' product[30].

It was read the link between color and the concentration of secondary compounds in tea plants (*Camellia sinensis*) and found three plant types of colored callus (white park and red) were produced from the tea bush using anti-browning treatments, and a comprehensive analysis of the phenolic substances of each was accompanied[31]. The results showed a clear difference in the accumulation of phenolic acids between the different colors; red callus contained higher amounts of compounds such as epicatechin, catechin, p-coumaric acid, and protocatechuic acid than white or green callus. Although the impact of environmental conditions is mentioned in the study on the chicory plant (*Cichorium endive*)[32], the use of red lighting considerably promoted the accumulation of flavonoids and phenolic compounds, as well as more intense color in the callus. This experiment demonstrates how physical characteristics and chemical content are related. Also, another study showed that the accumulation of cyanidin-3-glucoside, a flavonoid was greater in colored callus than in uncolored callus, confirming the theory that secondary metabolite accumulation in plant cells is reflected in color[33].

## 5. Conclusion

The accumulation of active glycosides and the color of the callus were both impacted by the application of several plant growth regulators on *T. gesneriana* callus formation. The combination of 2,4-D (1 mg/L) and TDZ (1 mg/L) dramatically produced red-colored callus with the greatest amounts of 6-Tuliposide A and B, indicating increased formation of secondary metabolites. However, the cream-colored callus from the 2,4-D (1.5 mg/L) and BA (1.5 mg/L) treatments had the lowest levels of tulipalin A and B. These results demonstrate a robust relationship between metabolic activity and callus hue, providing important information for enhancing in vitro conditions for tulip bioactive chemical production. Many researchers believe that callus color can serve as an indirect visual signal to estimate secondary metabolite accumulation, and the results of this study support this theory. This observation provides a simple and useful tool in the early stages of plant extract production within tissue culture programs, without the need for immediate chemical analysis.

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## Conflict of Interest

The authors declare that they have no conflicts of interest.

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