Callus Induction and Enhancing the Production of Biomass and Pharmaceutical Components of *Thymus decussatus* as an Endangered Medicinal Plant in Egypt

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ABSTRACT:

Thymus decussatus is a perennial herbaceous endangered medicinal plant belongs to family *Lamiaceae*. *Thymus* species are considered the most popular herbs in Mediterranean region due to their medicinal and nutritional values. However, at the same time they are also threatened due to intensive harvesting. The protocol for inducing callus in *Thymus decussatus* was created and executed on Murashige and Skoog (MS) medium. This medium was supplemented with various concentrations of 6-Benzylaminopurine (BAP) in combination with Naphthalene acetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D). The callus induction was performed using different types of explants derived from plants growing in vitro. The maximum callus induction percentage of 100%, was achieved by culturing stem segment explants on MS medium supplemented with 1.0 mg-1 BAP and 0.25 mgl-1 2,4-D, resulting in a fresh callus weight of 16.72 g and a dry weight of 0.37 g. The study investigated the impact of biotic (yeast extract) and abiotic (salicylic acid) elicitation on callus productivity and the accumulation of secondary metabolites. The best concentration of yeast extract (150 μM) resulted in the highest callus fresh weight of 10.72 g, while the best concentration of salicylic acid (250 μM) produced the highest callus fresh weight of 15.01 g after 30 days of culturing. Salicylic acid (SA) showed superiority in promoting the development of callus, as evidenced by increased fresh and dry weight. Both elicitors gave positive results in enhancing secondary metabolites accumulation and increased the quantity of some phenolic compounds which have a significant biological effects and benefits for human health.

Keywords: callogenesis; Thyme; elicitor; secondary metabolites; salicylic acid; *lamiaceae*; yeast extract

INTRODUCTION

Egypt has a significant biodiversity, with 529 therapeutic species, 60 endangered plants, and 13 pharmacopoeias (Boulos, 2009; Eissa et al., 2014), with natural diversity ranging from deserts to coasts (the Mediterranean and Red coasts), Nile Delta, Nile River, Depressions, Oases, and Mountains (Zahran and Willis, 2008). The Sinai Peninsula, notably Saint Katherine Protectorate is the most viable source of traditional herbs in Egypt. Sinai plant species include unique metabolites with significant pharmacological effects (Batanouny et al., 1999; Elshamy et al., 2019). Lamiaceae is regarded an important family, featuring different aromatic plants comprised 236 genera and 6900-7200 species (Harley et al., 2004; Heywood et al., 2007).

Thymus genus is among the most significant aromatic plant with therapeutic properties (Stahl-Biskup and Sáez, 2002; Marin et al., 2008). Also, the eighth-most abundant genus in Lamiaceae family. It offers a natural supply of monoterpene phenolic oils, oleoresins, fresh and dried plants (Lawrence and Tucker, 2002), and have been employed for numerous centuries in traditional medicine (Stahl-Biskup, 2002) because of their antibacterial, antiviral, antimicrobial, and antioxidative qualities (Reddy et al., 2014). In recent decades, human activities have resulted in the consumption of conventional food supplements, overharvesting of fuel and pharmaceutical health products, constrained range distribution, a low rate of natural reproduction, mine overexploitation, and persistent overgrazing. T. decussatus is on the verge of extinction and is considered an extremely vulnerable species in Egypt (Jalili and Jamzad, 1999).

Secondary metabolites synthesized by many plant organs, including roots, stems, leaves, and other above ground structures, are utilized for the formulation of pharmaceuticals (Pan, 2014; Raomai et al., 2015). Plant secondary metabolites are typically extracted from wild plants, a practice that often results in excessive utilization and thus endangers their survival (Song et al., 2014). Besides, callus produced in vitro has the ability to synthesize secondary metabolites that are similar to those present in the parent plant so that, callus culture can provide sustainable therapeutic compounds and could also be a new method by treating cultured cells as individual organisms instead of using the whole plant thus protecting it from extinction (Janarthanam et al., 2010; Efferth, 2019). There are many attempts towards an efficient in vitro callus induction protocol for an endangered and valuable medicinal plant, (Kakalis et al., 2023) recorded that different plant growth regulators, light conditions, and ascorbic acid supplements were assessed for optimizing callus induction from oregano leaves. Also, according to (Bakhtair et al., 2016) callus induction of T. persicus was performed on MS medium supplemented with different concentrations of NAA and 2,4-D, alone or in combination with BAP and Kin.

Secondary metabolites synthesized and accumulated within plants act as defense mechanisms against biotic factors like herbivores, fungi, bacteria or viruses as well as abiotic factors such as UV radiation, drought stress, high/low temperature etc. (Kaur and Pati, 2018). Therefore, there is need to increase the yield of secondary metabolite production in callus culture through specific elicitation since this is a powerful technique for enhancing biosynthesis and accumulation of secondary metabolites in vitro based tissue cultures of plants (Ramakrishna and Ravishankar, 2011; Wang and Wu, 2013). SA, also known as 2-hydroxybenzoic acid, it is a plant phenolic compound that an effective inducer which activates genes responsible for defence mechanism in plants. SA can considerably promote the synthesis of many different types of secondary compounds such alkaloids, terpenoids, phenolics among others phytoalexins too (Vlot et al., 2008). According to (Alvarez et al., 2000) SA greatly enhanced production levels of alkaloids in the hairy root culture derived from Brugmansia x candida Pers. Similarly (Mendhulkar et al., 2013) introduced term "salicylic acid" after finding its benefits towards total flavonoids content raised during suspension culture of Andrographis paniculata Burm.f. Also, yeast extract is a set of compounds that have a beneficial effect on plant growth, productivity, microelement composition, and the content of phytohormones and other plant metabolites (Naik and Al-Khayri, 2016; Halder et al., 2019). According to Zhao etal., 2014, elicitation with yeast polysaccharide effectively increased hairy root growth and Flavonoids (rutin and quercetin) production of Fagopyrum tataricum in aconcentration dependent manner by the stimulation of the phenylpropanoid pathway.

The aim here is therefore to develop an in vitro culture technique that will induce callus formation from different explant types of T. decussatus and in vitro stimulating secondary metabolite production using various elicitors which are alternative biotechnology methods for producing potent secondary metabolites required for future pharmacological investigations with T. decussatus as the plant material of choice where natural resources are scarce.

MATERIALS AND METHODS

Plant materials: Seeds of *T. decussatus* were collected from a wild population in the mountain tops in Saint Katherine Protectorate, South Sinai, Egypt and washed under tap water for an hour then soaked in 70% ethanol for 30 seconds. Seeds were surface sterilized with 0.75% commercial NaOCl solution for 15 minutes. All seeds were rinsed for 4-5 times with sterile double distilled water and cultured on MS medium (Murashige and Skoog, 1962) without any plant growth regulators (PGRs) for seed germination. After two months of germination the whole leaves were collected while the stems were cut into sections of 1-1.5 cm-long segments. All parts were cultured on MS medium supplemented with different concentrations and combinations of (PGRs) for callus induction.

Nutrient medium and culture conditions: The nutrient medium used in this study was MS culture medium supplemented with 3% sucrose, 0.5% phytagel, and pH was adjusted to 5.8 ± 2 using 1.0 N sodium hydroxide (NaOH) or 1.0 N hydrochloric acid (HCl). Then, all jars were closed with autoclavable polypropylene caps and autoclaved at a temperature of 121°C for 20 minutes under 1.1 kg/cm2. The laminar airflow chamber was exposed to ultraviolet (UV) light for 30 minutes to sterilize the surface of the working area.

Callogenesis: The callus was induced from leaves and stem segments on MS medium supplemented with various concentrations of (BAP) (0.0, 0.5, 1.0, 2.0, 3.0 mgl-1) in combination with different concentration of (NAA) (0.5, 1.0 ,2.0 mgl-1) or with different concentration of 2.4-D (0.25, 0.5, 1.0 mgl-1). Callus induction took place in 250 ml glass jars, containing 50 ml of the previous described medium, sealed with plastic caps. All the cultures were incubated in a culture room maintained at a temperature of 25 ± 2°C under 16 h light and 8 h dark using cool-white fluorescent lamps (Philips, 58W, Holland). Results were recorded after 21 days of the third subculture (fresh, dry weight and morphological features of callus). The cultured tissues were carefully taken from the culture vessel and cleaned from phytagel particles that had adhered at the point of contact. After that, the tissue was placed in a pre-weighted petri dish and the weight was determined using a single pan digital balance of fresh weight of callus which presented as gram (g). The tissues were oven-dried at 50°C to a consistent weight on the same petri dishes for estimation of their dry weight as g. Percentage of callus induction frequency was detected according to the following equation of (Mostafiz and Wagiran, 2018).

Callus induction frequency (%) = (Number of explants induced callus / Number of explants cultured) × 100. Also, the colour, uniformity and texture of callus were recorded .

Elicitation: Two elicitors (yeast extract and salicylic acid) were used for the enhancement of productivity of biomass and secondary metabolites accumulation of callus culture. Yeast extract was dissolved in double sterile distilled water with different concentrations (0.0, 50, 100, 150, 200, 250 and 300 mgl-1) and SA was dissolved in pure ethanol and were added with different concentrations (0.0, 50, 100, 150, 200, 250 and 300 μM) through a micro filter of 0.22 μ pore size to MS basal medium fortified with 1.0 mgl-1 BAP in combination with 0.25 mgl-1 2,4-D. For elicitation purposes, equal callus fragments (1.0 g) of friable white callus, grown in vitro for two months, were transferred to the previous medium supplemented separately with different concentrations of yeast extract and salicylic acid (SA). Increasing in callus growth represented by fresh and dry weight were recorded after 15 and 30 days of incubation under controlled conditions and compared to callus growth on control medium (without elicitors).

Preliminary phytochemical screening

The powdered samples of the produced calli of T. decussatus were screened for phytochemical constituents

Extraction of phenolic compounds

The callus extracts were prepared according to the method of (Castro et al., 2016). Briefly, 1.0 g of callus from each treatment was dried in an oven at 50°C for 24 hours, and then were soaked in 5 ml diethyl ether for 24 h. In order to prevent the evaporation of diethyl ether, the vials were kept closed and extraction was performed in a cold room. After 24 hours, the extracts were poured in to clean vials, and the leaves were rinsed with another 2.5 ml diethyl ether, which was added to the initial extracts. After that adding 1 ml of 80% methanol to the remaining solid material, the extracts were filtered (0.22 μm pore size) into clean vials and prepared for injection to HPLC instrument .

HPLC analysis

Phenolic compound contents of the callus were determined using high performance liquid chromatography (HPLC) instrument. HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using Zorbax Eclipse Plus C8 column (4.6 mm x 250 mm i.d., 5 μm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate 0.9 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0 –1 min (82% A); 1-11 min (75% A); 11-18 min (60% A); 18-22 min (82% A); 22-24 min (82% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 5.0 μl for each of the sample solutions. The column temperature was maintained at 40 °C.

Experimental design and statistical Analysis: All experiments were conducted under controlled conditions with five replications. The statistical analysis was performed using one –way analysis of variance (ANOVA) by the general linear models (GLMs) approach in the Minitab 19 System. The least significant difference (LSD) approach was used for mean comparisons (Lesik, 2018).

RESULTS AND DISCUSSION

Effect of PGRs and explant type on callus induction

In this study, it was found that the selection between NAA or 2,4-D in combination with BAP, as well as the choice of explants (leaf or stem segment), are essential factors in callus induction experiments. Such conclusions may be drawn because various factors, including genotype, culture medium and its components, plant growth regulators (PGRs), and explant type, all influence the developmental systems of cell cultures, as reported by Pandey et al. (2013) and Abd El-Motaleb et al. (2023). Based on Table 1 the results showed that MS medium supplemented with BAP and NAA or 2,4-D were effective for inducing calli from stem segment explants. No callus formed on the control medium devoid of plant growth regulators (PGRs), indicating that these substances are required to induce callusing from stem segments.

The highest callus induction frequency was observed in MS medium supplemented with 1.0 mg L^{-1} BAP and 0.25 mg L^{-1} 2,4-D. In stem nodal segment explants, this combination resulted in a fresh weight of 16.72 g and a dry weight of 0.37 g. The induced callus was morphologically white, spongy, friable, and nodular (Table 1; Figure 1A). Similarly, for callus obtained from leaf explants, the same medium produced the highest fresh weight of 12.25 g and a dry weight of 0.27 g. The callus was white-brownish in color, compact, friable, and exhibited a nodular structure (Table 2; Figure 1B).

MS medium supplemented with 3.0 mgl-1 BAP and 2.0 mgl-1 NAA resulted in the lowest callus induction percentage (43.33%) for stem segment explants, with a fresh weight of 0.21 g and a dry weight of 0.02 g. The induced callus was morphologically white- green with a brownish color and had a compact, nodular texture (Table 1; Figure 2A). Similarly, for leaf explant, MS medium supplemented with 2.0 mgl-1 BAP and 1.0 mgl-1 NAA resulted in the lowest callus induction percentage (33.33%), with a fresh weight of 0.29 g and a dry weight of 0.016 g. The callus was morphologically green with brownish color and exhibited a compact, nodular texture (Table 2; Figure 2F).

Both types of selected plant tissue samples exhibited the highest rate of callus formation, aligning with findings from previous studies on various plant species, including Mentha spicata (Poovaiah et al., 2006) Artemisia pallens (Nathar and Yatoo, 2014), Lavandula angustifolia (Machado et al., 2014), T. hyemalis (Nordine et al., 2014) and T. persicus (Bakhtair et al., 2016). The production of calli and their characteristics (colour, texture, surface, and uniformity) varied significantly depending on the concentration and combination of PGRs. This result aligns with the findings of Abd El-Motaleb et al. (2023), who reported that different levels of growth regulators in the medium influenced the morphological characteristics of calli. Additionally, nodal segments were more effective than leaf explants in terms of callus induction percentage, as well as callus fresh and dry weight.

Callus produced from stem segment explants of T. decussatus cultured on MS medium supplemented with 1.0 mg l-1 BAP in combination with 0.25 mg l-1 of 2,4-D was superior to that produced from leaf explant cultured on the same medium composition (Figure 1A, B). This result aligns with the findings of Bakhtair et al. (2016), Nasrat et al. (2022), Razavizadeh et al. (2019), and Mahood et al. (2022), who reported that the stem segment was the most effective explant type for callus induction. Additionally, they observed that 2,4-D, as a strong auxin, promoted callus formation more effectively than other forms of auxin. This finding is consistent with the results obtained by Kakalis et al. (2023), who achieved a maximum callogenesis rate (100%) from oregano leaves cultured on MS medium supplemented with 0.5 mg l−1 2,4-D and 3 mg l−1 BAP. Furthermore, this result is in harmony with the findings of Abd El-Motaleb et al. (2023), who reported that the combination of 0.25 mg l−1 BAP with 2 mg l−1 2,4-D resulted in the highest callus percentage and fresh weight.

In general, the findings of this study strongly support and agree with those of Pandey et al. (2013), who reported significant differences in callus induction and plantlet regeneration in Psoralea corylifolia based on explant type and the composition of growth regulators the culture medium. Similarly, these results are consistent with those of Abd El-Motaleb et al. (2023), who demonstrated that varying concentrations of plant growth regulators (PGRs) in the medium induced morphological alterations in calli. Stem segments outperformed leaf explants in terms of callus induction percentage, fresh and dry weight, and initiation time, which is in agreement with the findings of Mahood et al. (2022). However, these results contrast with those reported by Tokgoz and Altan (2020) and Kakalis et al. (2023), who successfully induced callus formation using leaf explants.

Effect of elicitation on callus growth

The impact of biotic elicitation, achieved by adding yeast extract (YE), and abiotic elicitation, achieved by adding salicylic acid (SA), to the callus culture medium can be observed in Tables 3 and 4, as well as Figures 3, 4, and 5. These measurements specifically pertain to callus growth in terms of fresh and dry weight .

The type of elicitor, its concentration, and the treatment schedule all have a significant impact on callus growth and secondary metabolite production. As shown in Table 3, the combination of dosage and incubation period had a synergistic effect on callus mass formation in T. decussatus. After 15 days of culture, the lowest concentration of YE (50 mgl-1) had the greatest impact on callus growth compared to both the control medium and higher concentrations of YE .

As the concentration of YE increased to its highest level (300 mgl-1), callus development gradually decreases to a minimum. After 15 days of culture, treatment with 50 mgl-1 YE resulted in the maximum callus growth, increasing fresh weight from 1.687 g to 4.02 g and dry weight from 0.167 g to 0.223 g. This value was significantly higher than all other treatments. After a 30-day period, the experiment involved feeding the culture with various concentrations of YE. The findings indicated that YE treatment led to a significant increase in callus growth. This was evident from the rise in fresh weight from 5.217 g in the control medium to 10.72 g, as well as the increase in dry weight from 0.23 g in the control medium to 0.337 g in callus treated with 150 mgl-1 YE (Figure 3) .

Table (4) presents the influence of different SA concentrations and incubation times on callus growth of T. decussatus. The study found that higher concentrations of SA (200, 250 μM) had the most significant impact on callus growth after 15 days of culture, outperforming both the control medium and lower SA concentrations. The highest callus growth after 15 days of culture was observed with 250 μM SA, which increased the fresh weight from 1.686 g to 2.63 g and the dry weight from 0.166 g to 0.216 g. All other treatments resulted in significantly lower values compared to this.

After 30 days of culture, the results indicated that treatment with SA led to a significant increase in callus growth. This increase was accompanied by a notable rise in fresh weight of callus, from 7.78 g in the control medium to 15.01 g. Additionally, the dry weight of calli treated with 250 μM SA increased from 0.336 g in the control medium to 0.47 g (Figure 4). Based on these findings, it can be concluded that the type of elicitor (salicylic acid and yeast extract), its concentration and the elicitation period play crucial roles in inducing various responses related to cell growth parameters, particularly biomass yield. Furthermore, varying concentrations of elicitors significantly influence cell development. However, excessive concentrations of elicitors can trigger a hypersensitive response, leading to cell death, emphasizing the necessity of an optimal elicitor level for effective induction. These findings are in agreement with those reported by Al-Khayri and Naik (2020) and Mahood et al. (2022). In conclusion, stem segment explants of T. decussatus exhibited the best results for in vitro callus induction when cultured on MS medium supplemented with 1.0 mgl-1 BAP and 0.25 mgl-1 2,4-D. The type of elicitor and incubation period significantly influenced callus growth, as reflected in fresh and dry weight measurements. After 30 days of incubation, SA at 250 μM proved to be more effective in promoting T. decussatus callus biomass than yeast extract.

Effect of biotic elicitation (YE) on the productivity of callus phenolic compounds

Elicitation is one of the most effective and widely used biotechnological approaches for inducing the synthesis of novel secondary metabolites, enhancing biosynthesis, and promoting the accumulation of secondary metabolites in in vitro plant tissue culture (Ramakrishna and Ravishankar, 2011; Wang and Wu, 2013).

For this investigation, calli derived from the medium containing the optimal elicitor concentration (150 mgl-1 YE), which resulted in the highest fresh and dry weight of callus, were selected and compared to callus grown on the control medium (without elicitor) and the mother plant in terms of active constituent content .

The HPLC analysis in Graphs 1, 3, and 4 and Table 5 highlights the impact of yeast extract (YE) as a biotic elicitor on secondary metabolite accumulation in T. decussatus callus. YE treatment significantly enhanced key bioactive compounds. Chlorogenic acid increased from 5.92 μg mL^{-1} in control callus and 8.03 μg mL^{-1} in the mother plant to 10.11 μg mL^{-1}. Ellagic acid rose from 0.67 μg mL^{-1} in control callus and 1.25 μg mL^{-1} in the mother plant to 2.76 μg mL^{-1}. Daidzein increased from 0.62 μg mL^{-1} in control callus and 1.31 μg mL^{-1} in the mother plant to 4.34 μg mL^{-1} with YE treatment.

Chlorogenic acid, an ester of caffeic acid and (-)-quinic acid, plays a key role in lignin biosynthesis (Boerjan et al., 2003), essential for plant structure and defense. The term "chlorogenic acids" refers to a group of polyphenols, including caffeic, ferulic, and p-coumaric acids, along with quinic acid (Clifford et al., 2003). These compounds are known for their antioxidant properties and role in plant metabolism and human health.

Ellagic acid, a polyphenol found in many fruits and vegetables, is being studied for therapeutic uses such as treating Follicular Lymphoma, preventing brain injury in neonates, improving cardiovascular function, and treating solar lentigines (age spots). Its antioxidant and anti-proliferative effects make it a promising candidate for pharmaceutical and dermatological applications.

Daidzein, a naturally occurring isoflavone, is synthesized through the phenylpropanoid pathway, serving as a signalling molecule and plant defence compound (Jung et al., 2000). Research suggests it helps alleviate menopausal symptoms, prevent osteoporosis, lower cholesterol, and reduce the risk of hormone-related cancers and heart disease. Despite its benefits, daidzein and puerarin have low water solubility and poor bioavailability, limiting their effectiveness in medical and dietary use. Researchers are exploring ways to improve their absorption for better therapeutic potential (Wang et al., 2022).

The current study revealed that yeast extract (YE) enhanced the accumulation of several secondary metabolites in T. decussatus calli compared to the mother plant, notably chlorogenic acid, ellagic acid, and daidzein, all of which exhibit remarkable biological activities and health benefits for humans. This finding is in agreement with Cai et al. (2012), Ramirez-Estrada et al. (2016), and Singh et al. (2018), who reported that applying different elicitors to undifferentiated cells boosts secondary metabolite production. However, they also noted several limitations to this approach, including lower yields than those achieved in organ cultures, genetic and biosynthetic instability over long-term cultivation, and variable responses to identical elicitors. Ramirez-Estrada et al. (2016) further emphasized that yeast and fungal elicitors are widely employed to stimulate secondary metabolite production, especially in hairy root and cell cultures.

Numerous studies have documented the positive influence of YE on secondary metabolite synthesis, highlighting its potential to enhance both plant and cell growth by promoting biomass accumulation. For example, Krstić-Milošević et al. (2017) demonstrated that using YE as a biotic elicitor significantly increased root growth and biomass in Gentiana dinarica. Similarly, Bayraktar et al. (2016) employed YE to enhance biomass production in Stevia rebaudiana, attributing the improvement to increased growth-promoting activity. These findings support the use of YE as a practical elicitor in plant tissue culture systems.

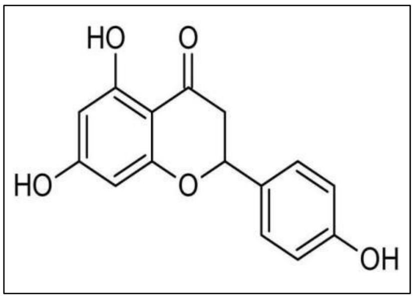
Effect of abiotic elicitation (SA) on productivity of callus phenolic compounds

Salicylic acid (SA) is a phenolic compound derivative known as 2-hydroxybenzoic acid. It is classified as a plant hormone and plays a major role in regulating plant growth and development, as reported by Raskin (1992) and Taguchi et al. (2001). In the present study, calli obtained from the medium containing the most effective concentration of SA (250 μM), which resulted in the highest fresh and dry weights, were selected for comparison. These calli were analyzed alongside those grown on the control medium (without elicitor) and the mother plant to evaluate differences in the content of active constituents.

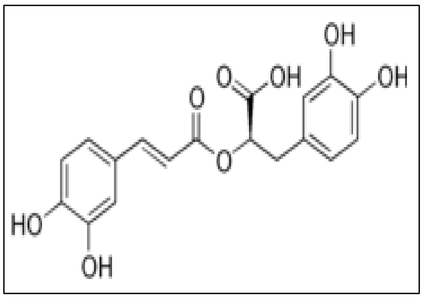
The HPLC analysis in Graphs 2, 3, and 4 and Table 6 presents findings on the impact of salicylic acid (SA) as an abiotic elicitor on the content of secondary metabolites in T. decussatus callus. The elicitation effect of SA showed positive outcomes by significantly enhancing metabolite levels. Chlorogenic acid increased from 5.92 μg mL^{-1} in callus grown on the control medium and 8.03 μg mL^{-1} in the mother plant to 21.23 μg mL^{-1} after SA treatment. Naringenin rose from 0.18 μg mL^{-1} in the mother plant and 0.38 μg mL^{-1} in control callus to 0.96 μg mL^{-1} in SA-elicited callus. Rosmarinic acid content increased significantly, from 96.26 μg mL^{-1} in callus grown in control medium and 465.78 μg mL^{-1} in the mother plant to 815 μg mL^{-1} with SA elicitation. Daidzein also showed an increase, rising from 0.62 μg mL^{-1} in control callus and 1.31 μg mL^{-1} in the mother plant to 4.62 μg mL^{-1} after SA treatment. These results confirm that SA has a strong elicitation effect on the biosynthesis of various phenolic compounds in plant cell cultures.

Naringenin is a flavanone that belongs to the flavonoid group of polyphenols (Felgines et al., 2000). It has demonstrated several biological activities, including anti-inflammatory, antioxidant, and skin-healing effects (Wang et al., 1999; Karuppagounder et al., 2016; Martinez et al., 2016; Al-Roujayee, 2017). Naringenin is also used as a cosmetic ingredient and dietary supplement (Rebello et al., 2020).

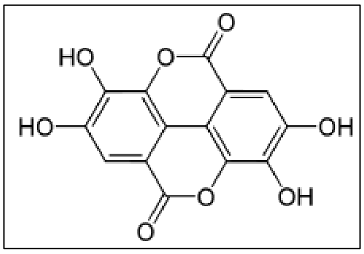
Rosmarinic acid is a caffeic acid ester and a naturally occurring phenolic compound found in many plants of the Lamiaceae family (Nadeem et al., 2019). It offers a wide range of biological activities, including antiviral, antioxidant, antibacterial, anticancer, antidiabetic, anti-aging, cardioprotective, nephroprotective, hepatoprotective, antidepressant, antiallergic, and anti-inflammatory effects.



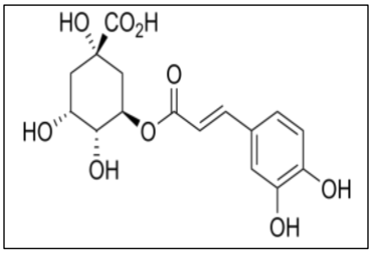
Naringenin



Rosmarinic acid



Daidzein



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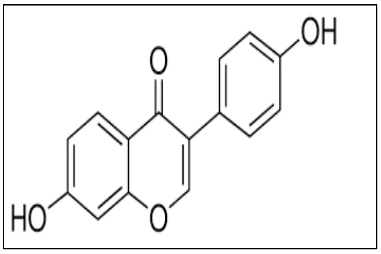


Ellagic acid

The current study demonstrated that salicylic acid (SA) stimulated the accumulation of certain phenolic compounds in T. decussatus calli, particularly rosmarinic acid, which nearly doubled in concentration compared to its level in the mother plant. This result aligns with the findings of Mendoza et al. (2018), who reported that SA (300 µM) and MeJA (3 µM) increased the levels of phenolic and flavonoid compounds, suggesting the inducer role of these elicitors in the phenylpropanoid metabolic pathway. Additionally, the present findings are consistent with those of Figueroa Perez et al. (2014) and Kandoudi and Nemeth-Zamborine (2022), who observed that SA treatment of peppermint increased total phenolic and flavonoid content at all tested concentrations compared to the control.

CONCLUSION

The effectiveness of callus formation depends on the type of growth regulators used and the source of the explant. This study showed that T. decussatus explants respond differently to growth regulators in the culture medium, with stem segments performing best. Among the auxins tested, 2,4-D was the most effective for inducing callus when combined with BAP. The highest callus induction rate (100%) was achieved using stem segment explants on MS medium supplemented with 1.0 mg L^{-1} BAP and 0.25 mg L^{-1} 2,4-D.

Additionally, yeast extract as a biotic elicitor and salicylic acid as an abiotic elicitor significantly enhanced the accumulation of secondary metabolites in the callus. Based on these findings, the use of callus culture is recommended as a modern biotechnological approach for the production of phytopharmaceutical compounds.

REFERENCES

Abd El-Motaleb, M., Abd El-Hameid, A.R., Helmy, W.A., Ewais, E,A., Abdel-Hady, M.S. 2023: Establishment of callogenesis and plant regeneration protocols for endemic *Origanum syriacum* ssp. Sinaicum. J. Crop Sci. and Biotech.

Al-Khayri, J.M., Naik, P.M. 2020: Elicitor-Induced Production of Biomass and Pharmaceutical Phenolic Compounds in Cell Suspension Culture of Date Palm (*Phoenix dactylifera* L.). J. Molecules, 25(20),4669.

Al-Roujayee, A.S. 2017: "Naringenin improves the healing process of thermally-induced skin damage in rats". The Journal of International Medical Research, 45 (2): 570–582.

Alvarez, P.S., Spollansky, T.C., Giulietti, A.M. 2000: The influence of different biotic and abiotic elicitors on the production and profile of tropane alkaloids in hairy root cultures of *Brugmansia candida*. Enzyme and Microbial Technology,26(2-4): 254-258.

Bakhtiar, Z., Mirjalili, M.H., Sonboli, A. 2016: *In vitro* callus induction and micropropagation of *thymus persicus* (lamiaceae), an endangered medicinal plant. Crop Breed. J. Appl. Biotechnol., 16 (1): 48-54.

Batanouny, KH., Aboutabl, E., Shabana, M.F.S. 1999: Wild Medicinal Plants in Egypt. An Inventory to Support Conservation and Sustainable Use; The Palm Press: Cairo, Egypt.

Bayraktar, M., Naziri, E., Akgun, I.H., Karabey, f., Ilhan, E., Akyol, B., Bedir, E., Gurel, A. 2016: Elicitor induced stevoside production, *in vitro* shoot growth, and biomass accumulation in micropropagated *Stevia rebaudiana.* Plant Cell, Tissue and Organ Culture (PCTOC), volume (127): pp 289-300.

Boerjan, W., Ralph, J., Baucher, M. 2003: Lignin biosynthesis. Annual Review of Plant Biology, 54: 519–546.

Boulos, L. 2009: Flora of Egypt Checklist; Al-Hadara Publishing: Cairo, Egypt, pp. 198–201.

Cai, Z., Kastell, A., Knorr, D., Smetanska, I. 2012: Exudation: an expanding technique for continuous production and release of secondary metabolites from plant cell suspension and hairy root cultures. Plant Cell Rep., 31, 461–477.

Castro, A.H.F., Braga, K.D.Q., Sousa, F.M.D., Coimbra, M.C., Chagas, R.C.R. 2016: Rev. Ciˆenc Agron., 47(1), 143–151.

Clifford, M.N., Johnston, K.L., Knight, S., Kuhnert, N. 2003: "Hierarchical Scheme for LC-MSn Identification of Chlorogenic Acids". Journal of Agricultural and Food Chemistry, 51 (10): 2900–2911. doi:10.1021/jf026187q.

Efferth, T. 2019: Biotechnology applications of plant callus cultures. J. Engineering, 5:50–59.

Eissa, T., Palomino, O., Carretero, M., Gómez-Serranillos, M. 2014: Ethnopharmacological study of medicinal plants used in the treatment of CNS disorders in Sinai Peninsula, Egypt. J. Ethnopharmacol, 151: 317–332.

Elshamy, A.I., Abd El-Gawad, AM., El Gendy, A.E-N.G., Assaeed, A.M. 2019: Chemical characterization of *Euphorbia heterophylla* L. essential oils and their antioxidant activity and allelopathic potential on *Cenchrus echinatus* L. J. Chem. Biodivers. , 16, e1900051.

Felgines, C., Texier, O., Morand, C., Manach, C., Scalbert, A., Régerat, F., Rémésy, C. 2000: "Bioavailability of the flavanone naringenin and its glycosides in rats". American Journal of Physiology. Gastrointestinal and Liver Physiology, 279 (6): G1148–G1154.

Figueroa Perez, M.G., Rocha-Guzman, N.E., Mercado-Silva, E., 2014: Effect of chemical elicitors on peppermint (*Mentha piperita*) plants and their impact on the metabolite profile and antioxidant capacity of resulting infusions. Food Chem, 156: 273-278.

Halder, M., Sarkar, S., Jha, S. 2019: Elicitation: a biotechnological tool for enhanced production of secondary metabolites in hairy root cultures, Eng. Life Sci., vol. 19, no. 12, pp. 880–895.

Harley, R.M., Atkins, S., Budantsev, A.L., Cantino, P.D., Conn, B.J., Grayer, R.J., Harley, M.M., de Kok, R.P., Morales, R., Paton, A.J., 2004: Labiatae. In the families and genera of vascular plants, Kubitzki K, Ed; J. Springer: Berlin, Germany, 7: 2275–2283.

Heywood, V.H., Brummitt, R., Culham, A., Seberg, O. 2007: Flowering Plant Families of the World; Firefly Books: Ontario, ON, Canada, Volume 88.

Jalili, A., Jamzad, Z. 1999: Red data book of Iran: A preliminary survey of endemic, rare and endangered plant species in Iran. Research Institute of Forest and Rangelands Publication, Tehran, 748p.

Janarthanam, B., Gopalakrishnan, M., Sekar, T. 2010: Secondary metabolite production in callus cultures of *Stevia rebaudiana* Bertoni. Bangladesh J. Sci Ind Res., 45 : 243–248.

Jung, W.S., Yu, O., Lau, C.S.M., O'Keefe, D.P., Odell, J., Fader, G., McGonigle, B. 2000: Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. Nature Biotechnology, 18 (2): 208–212. doi:10.1038/72671.

Kakalis, A., Tsekouras, V., Mavrikou, S., Moschopoulou, G., Kintzios, S., Evergetis, E., Iliopoulos, V., Koulocheri, S.D., Haroutounian, S.A. 2023: Farm or Lab? A Comparative Study of Oregano's Leaf and Callus Volatile Isolates Chemistry and Cytotoxicity. Plants, 12, 1472.

Kandoudi, W., Nemeth-Zamborine, E. 2022: Stimulating secondary compound accumulation by elicitation: Is it a realistic tool in medical plants *in vivo*. Phytochem Rev, 21: 2007-2025.

Karuppagounder, V., Arumugam, S., Thandavarayan, R.A., Sreedhar, R., Giridharan, V.V., Pitchaimani, V. 2016: "Naringenin ameliorates skin inflammation and accelerates phenotypic reprogramming from M1 to M2 macrophage polarization in atopic dermatitis NC/Nga mouse model". Experimental Dermatology, 2016; 25(5): 404–407.

Kaur, K., Pati, P.K. 2018: Stress-induced metabolite production utilizing plant hairy roots, in: Srivastava V., Mehrotra S., Mishra S. (Eds.), Hairy Roots- An Effective Tool of Plant Biotechnology, J. Springer, Singapore: pp. 123–145.

Krstić-Milošević, D., Janković, T., Uzelac, B., Vinterhalter, D., Vinterhalter, B. 2017: Effect of elicitors on xanthone accumulation and biomass production in hairy root cultures of *Gentiana dinarica*. Plant Cell, Tissue and Organ Culture (PCTOC), Volume (130): pp 631-640.

Lawrence, B.M., Tucker, A.O. 2002: The genus *Thymus* as a source of commercial products. In Stahl-Biskup E and Sáez F (eds) Thyme, the genus *Thymus*. Taylor and Francis, London, p. 252-262.

Lesik, S.A. 2018: Applied statistical inference with MINITAB®. CRC Press, Boca Raton.

Machado, M.P., Silva, A.L.L., Biasi, L.A., Deschamps, C.B., Filho, J.C., Zanette, F. 2014: Influence of calcium content of tissue on hyperhydricity and shoot-tip necrosis of *in vitro* regenerated shoots of *Lavandula angustifolia* Mill. J. Brazilian Archives of Biology and Technology, 57: 636-643.

Mahood, H.E., Sarropoulou, V., Tzatzani, T.T. 2022: Effect of explant type (leaf, stem) and 2,4-D concentration on callus induction: influence of elicitor type (biotic, abiotic), elicitor concentration and elicitation time on biomass growth rate and costunolide biosynthesis in gazania (*Gazania rigens*) cell suspension cultures. J. Bioresources and Bioprocessing.

Marin, K.A., Bohanek, J.G., Fivush, R. 2008: Positive effects of talking about the negative: Family narratives of negative experiences and preadolescents' perceived competence. Journal of Research in Adolescence. 18: 573–593.

Martinez, R.M., Pinho-Ribeiro, F.A., Steffen, V.S.;, Silva, T.C., Caviglione, C.V., Bottura, C. 2016: "Topical Formulation Containing Naringenin: Efficacy against Ultraviolet B Irradiation-Induced Skin Inflammation and Oxidative Stress in Mice". PLOS One, 11 (1): e0146296.

Mendhulkar, V.D., Moinuddin, M., Vakil, A. 2013: Elicitation of flavonoids by salicylic acid and Penicillium expansum in *Andrographis paniculata* (Burm. f.) Nees. J. cell culture. Research in Biotechnology, 4(2): 1-9.

Mendozaa, D., Cuaspuda, O., Ariasa, J.P., Ruizc, O., Arias, M. 2018: Effect of salicylic acid and methyl jasmonate in the production of phenolic compounds in plant cell suspension cultures of *Thevetia peruviana. Biotechnology Reports*,19

Mostafiz, S., Wagiran, A. 2018: Efficient callus induction and regeneration in selected Indica rice. J. Agronomy, 8(5): 77.

Murashige, T., Skoog, F. 1962: A revised medium for rapid growth and bioassays with tobacco tissue cultures. J. Plant Physiol, 15: 473–497.

Nadeem, M., Imran, M., Gondal, T.A., Imran, A., Shahbaz, M., Amir, RM., 2019: "Therapeutic Potential of Rosmarinic Acid: A Comprehensive Review. Appl. Sci., 9(15), 3139.

Naik, P.M., Al–Khayri, J.M. 2016: Abiotic and biotic elicitors–role in secondary metabolites production through in vitro culture of medicinal plant, in: Shanker A. K., Shanker C. (Eds.), Abiotic and Biotic Stress in Plants-Recent Advances and Future Perspectives, InTech, Rijeka , pp. 247–277.

Nasrat, M.N., Sakimin, S.Z., hakiman, M. 2022: Phytochemicals and Antioxidant Activities of Conventionally Propagated Nodal Segment and *In Vitro*-Induced Callus of *Bougainvillea glabra* Choisy Using Different Solvents. J. *Horticulturae*, *8*(8), 712.

Nathar, V.N., Yatoo, G.M. 2014: Micropropagation of an antidiabetic medicinal plant, *Artemisia* pallens. Turkish J. Botany, 38: 491-498.

Nordine, A., Tlemcani, C.R., El-Meskaoui, A. 2014: Regeneration of plants through somatic embryogenesis in *Thymus hyemalis* Lange, a potential medicinal and aromatic plant. J. *In Vitro* Cellular & Developmental Biology – Plant, 50: 19-25.

Pan, W. 2014: Bioactive compounds from Vitex leptobotrys. J Nat Prod, 77: 663–667.

Pandey, P., Mehta, R., Upadhyay, R. 2013: Effect of Explants Type and Different Plant Growth Regulators on Callus Induction and Plantlet Regeneration in *Psoralea corylifolia* L. International Journal of Research in Pharmaceutical and Biomedical Sciences, ISSN: 2229-3701

Poovaiah, C.R., Weller, S.C., Jenks, M.A. 2006: *In vitro* adventitious shoot regeneration of native spearmint using internodal explants. J. Hortscience, 2006; 41: 414-417.

Ramakrishna, A., Ravishankar, G.A. 2011: Influence of abiotic stress signals on secondary metabolites in plants. J. Plant Signal Behav., 6: 1720-1731.

Ramirez-Estrada, K., Vidal-Limon, H., Hidalgo, D., Moyano, E., Golenioswki, M., Cusidó, RM., Palazon, J. 2016: Elicitation, an Effective Strategy for the Biotechnological Production of Bioactive High-Added Value Compounds in Plant Cell Factories. J. *Molecules,* 21, 182.

Raomai, S., Kumaria, S., Kehie, M., Tandon, P. 2015: Plantlet regeneration of Paris polyphylla Sm. via thin cell layer culture and enhancement of steroidal saponins in mini-rhizome cultures using elicitors. Plant Growth Regul., 75:341–353

Raskin, l. 1992: Role of salicylic acid in plants. Ann Rev Plant Physiol Mol Biol., 43(1): 439-63.

Razavizadeh, R., Farahzadianpoor, F., Adabavazeh, F., Komatsu, S. 2019: Physiological and morphological analyses of *Thymus vulgaris* L. *in vitro* cultures under polyethylene glycol (PEG)-induced osmotic stress. J. *In Vitro* Cellular, Developmental Biology-Plant, 55 : 342-357.

Rebello, C.J., Beyl, R.A., Lertora, J.J., Greenway, F.L., Ravussin, E., Ribnicky, D.M., 2020: "Safety and pharmacokinetics of naringenin: A randomized, controlled, single-ascending-dose clinical trial". Diabetes, Obesity & Metabolism, 22 (1): 91–98.

Reddy, P., Kandisa, RV., Varsha, PV., Satyam, S. 2014: Review on *Thymus vulgaris* traditional uses and pharmacological properties. J. Medicinal & Aromatic Plants, 3: 164-166.

Singh, N.R., Rath, S.K., Behera, S., Naik, S.K. 2018: *In vitro* secondary metabolite production through fungal elicitation: an approach for sustainability, in: Prasad, R., Kumar, V., Kumar, M., Wang, S. (Eds.), Fungal Nanobionics: Principles and Applications, Springer, Singapore, pp. 215–242.

Stahl-Biskup, E. 2002: Thyme as a herbal drug-pharmacopoeias and other product characteristics. In Stahl-Biskup E and Sáez F (eds) Thyme, the genus *Thymus*. J. Taylor and Francis, London, p. 293-316.

Stahl-Biskup, E., Saez, F. 2002: Thyme. Taylor and Francis, London Sur.

Song, M.C., Kim, E.J., Kim, E., Rathwell, K., Nama, S.J., Yoon, Y.J. 2014: Microbial biosynthesis of medicinally important plant secondary metabolites. Nat Prod Res, 31: 1497–1509

Taguchi, G., Yazawa, T., Hayashida, N., Okazaki, M. 2001: Molecular cloning heterologous expression of novel glucosyltransferases from tobacco cultured cells that have broad substrate specificity and are induced by salicylic acid and auxin. Eur J Biochem., 268(14): 4086-94.

Tokgoz, H.B., Altan, F. 2020: Callus Induction and Micropropagation of *Lilium candidum* L. Using Stem Bulbils and Confirmation of Genetic Stability via SSR-PCR. International Journal of Secondary Metabolite, Vol. 7, No. 4: 286-296

Vlot, A.C., Klessig, D.F., Park, S.W. 2008: Systemic acquired resistance: the elusive signal(s). Current Opinion in Plant Biology,11(4): 436-442.

Wang, H., Nair, M.G., Strasburg, GM., Booren, A.M., Gray, J.I. 1999: "Antioxidant polyphenols from tart cherries (Prunus cerasus)". Journal of Agricultural and Food Chemistry, 47 (3): 840–844.

Wang, J.W., WU, J.Y. 2013: Effective elicitors and process strategies for enhancement of secondary metabolites production in hairy root cultures, in: Doran, P.M.(Ed.), Biotechnology of hairy root systems. Advances in biochemical engineering /Biotechnology, J. Springer Berlin, Heidelberg, pp. 55-89.

Wang, Y.C., Yang, M., Qin, J.J., Wa, W.Q. 2022: Interactions between puerarin/daidzein and micellar casein. Journal of Food Biochemistry, 46 (2): e14048.

Zahran, M.A., Willis, A.J. 2008: The Vegetation of Egypt; Springer Science & Business Media: Berlin, Germany, Volume 2.

Zhao, J.L., Zou, L., Zhang, C.Q., Li, Y.Y. 2014: Efficient production of flavonoids in Fagopyrum tataricum hairy root cultures with yeast polysaccharide elicitation and medium renewal process.Pharmacogn. Mag., 10, 234–240

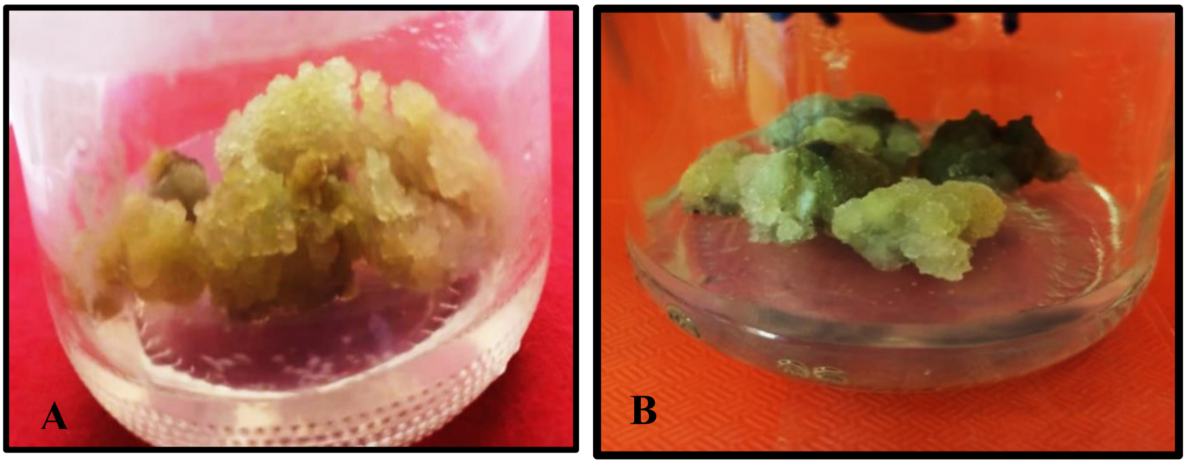


Figure 1: Callus formation of T. decussatus on MS medium fortified with 1.0 mgl-1 BAP +0.25mgl-1 2,4-D (A) Callus derived from stem segment explant; (B) Callus derived from leaf explant

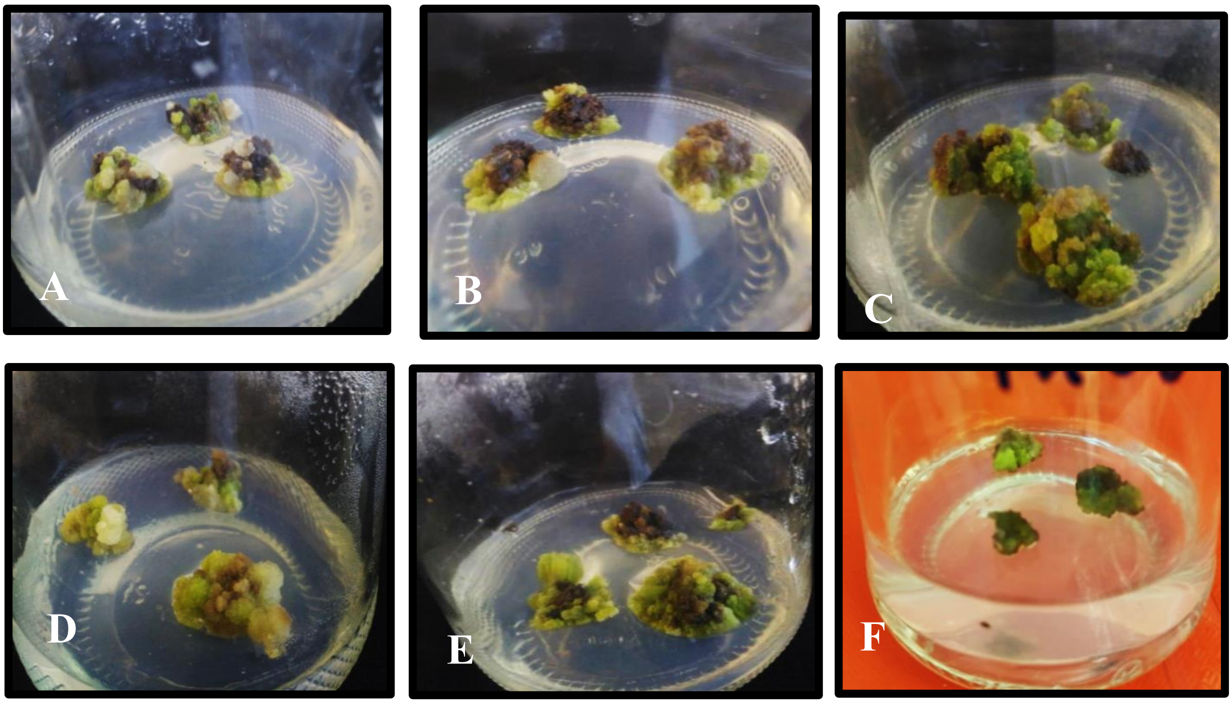


Figure 2: Callus formation of T. decussatus on MS medium supplemented with different concentrations of BAP and NAA. (A) 3.0 mg L^{-1} BAP + 2.0 mg L^{-1} NAA (stem nodal segment explants); (B) 1.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA (stem nodal segment explants); (C) 1.0 mg L^{-1} BAP + 2.0 mg L^{-1} NAA (stem nodal segment explants); (D) 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA (leaf explants); (E) 0.5 mg L^{-1} BAP + 2.0 mg L^{-1} NAA (leaf explants); (F) 2.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA (leaf explants).

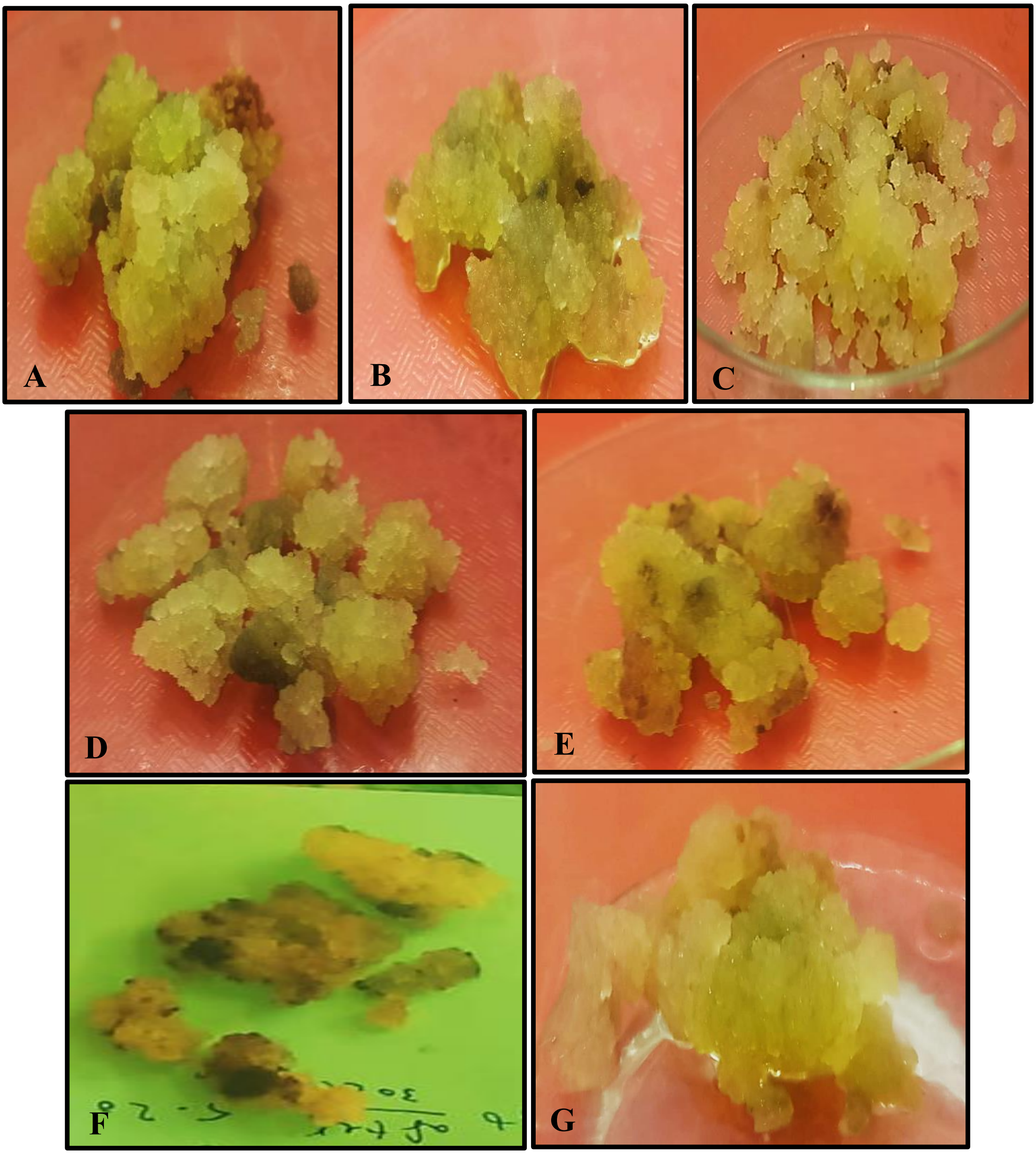


Figure 3: Effect of various concentrations of yeast extract (YE) on callus growth of *T. decussatus* after 30 days of incubation. (A) 50 mg L^{-1}; (B) 100 mg L^{-1}; (C) 150 mg L^{-1}; (D) 200 mg L^{-1}; (E) 250 mg L^{-1}; (F) 300 mg L^{-1}; (G) Control.

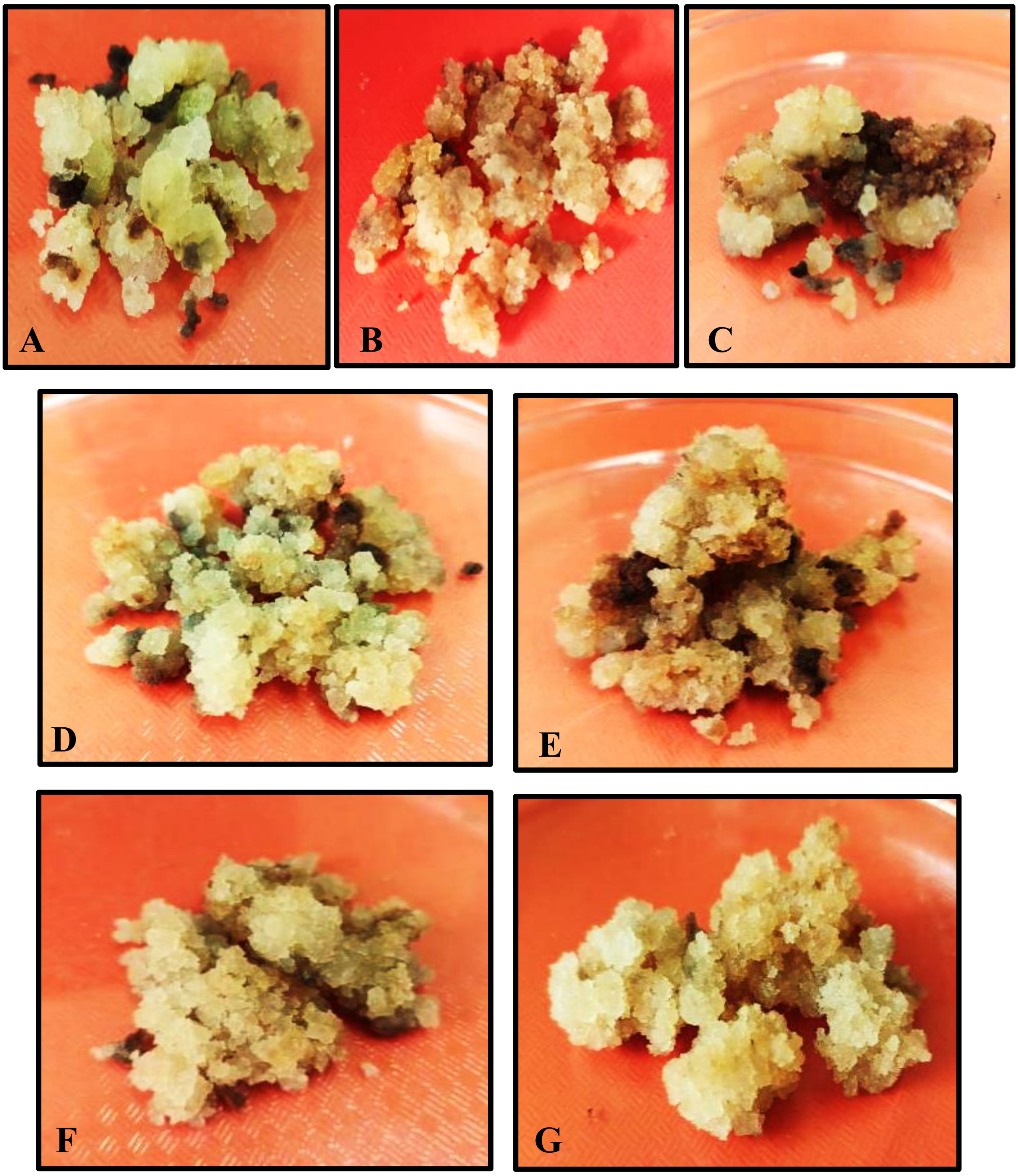


Figure 4: Effect of various concentrations of salicylic acid (SA) on callus growth of T. decussatus after 30 days of incubation. (A) 50 µM; (B) 100 µM; (C) 150 µM; (D) 200 µM; (E) 250 µM; (F) 300 µM; (G) Control.

Table 1: Effect of stem segment explant and various growth regulator treatments on fresh, dry weights and morphological character of *T. decussatus* calli.

PGRs treatment (mgl^{-1})	Callus induction frequency (%)	Fresh weight (g)	Dry weight (g)	Colour	Uniformity	Texture
Control (free MS medium)	0.00	0.00	0.00	-	-	-
(A)	66.67 ± 3.33^{cde}	0.36 ±0.22^{bc}	0.02 ±0.01^{b}	Brownish green	Patchy	Compact
(B)	83.33 ± 3.33^{abc}	0.81 ± 0.028^{bc}	0.05 ± 0.005^{b}	White green with brownish	Patchy	Compact friable
(C)	56.67 ±3.33^{def}	0.63 ± 0.09^{bc}	0.04 ± 0.01^{b}	Brownish green	Patchy	Compact friable
(D)	93.33 ± 6.67^{ab}	0.9± 0.38^{bc}	0.05 ± 0.02^{b}	Greenish white	Patchy	Compact
(E)	56.67 ±3.33^{def}	0.36 ± 0.05^{bc}	0.02 ± 0.002^{b}	Greenish white	Patchy	Spongy
(F)	53.33 ±3.33^{ef}	0.34± 0.08^{bc}	0.02 ± 0.004^{b}	green	Patchy	Compact friable
(G)	43.33 ± 3.33^{f}	0.21 ± 0.024^{c}	0.02 ± 0.00^{b}	White green with brownish	Patchy	Compact friable
(H)	100±0.00^{a}	16.72 ± 1.08^{a}	0.37 ± 0.018^{a}	White	Uniform	Spongy friable
(I)	76.67 ±3.33^{bcd}	2.207± 0.19^{b}	0.18± 0.00^{b}	Greenish white	Patchy	Compact friable
(J)	86.67 6.67^{abc}	1.33± 0.06^{bc}	0.18± 0.00^{b}	Greenish white	Patchy	Compact friable

Each value represents the mean ± SE. Means that do not share the same letter are significantly different. Results were recorded after 21 days of the third subculture. (A) 0.5 BAP+ 2.0 NAA; (B) 1.0 BAP+ 1.0 NAA; (C) 1.0 BAP+ 2.0 NAA; (D) 2.0 BAP+ 0.5 NAA; (E) 2.0 BAP+1.0 NAA; (f) 3.0 BAP+ 1.0 NAA; (G) 3.0 BAP+ 2.0 NAA; (H) 1.0 BAP+ 0.25 (2,4 D); (I) 1.0 BAP+ 0. 5 (2,4 D); (J) 1.0 BAP+ 1.0 (2,4 D)

Table 2: Effect of leaf explant and various growth regulator treatments on fresh, dry weight and morphological character of *T. decussatus calli*.

Hormonal treatment (mgl-1)	Callus induction Frequency (%)	Fresh weight (g)	Dry weight (g)	Colour	Uniformity	Texture
Control (free MS medium)	0.00	0.00	0.00	-	-	-
(A)	46.67 ± 3.33^{c}	0.49 ± 0.11^{b}	0.026 ± 0.007^{b}	White green with brownish	Patchy	Spongy friable
(B)	53.33 ± 3.33^{bc}	0.60 ± 0.11 ^{b}	0.032± 0.003^{b}	White green with brownish	Patchy	Spongy friable
(C)	53.33 ± 3.33^{bc}	1.003 ± 0.52^{b}	0.05 ± 0.03^{b}	White green with brownish	Patchy	Compact friable
(D)	73.33 ± 6.67^{ab}	0.53 ± 0.13^{b}	0.028 ± 0.004^{b}	Greenish white	Patchy	Spongy
(E)	33.33 ±3.33^{c}	0.29 ± 0.06^{b}	0.016 ± 0.00 ^{b}	Greenish white	Patchy	Compact
(F)	36.67 ± 3.33^{c}	0.58 ± 0.13 ^{b}	0.025 ± 0.006^{b}	White green with brownish	Patchy	Compact friable
(G)	36.67 ± 3.33^{c}	0.51 ±0.11 ^{b}	0.028 ±0.003^{bc}	Greenish white	Patchy	Compact
(H)	83.33 ± 3.33^{a}	12.25 ± 1.11^{a}	0. 27 ± 0.032^{a}	Brownish white	Patchy	Compact
(I)	70 ± 5.77^{ab}	1.81 ± 0.17^{b}	0.09 ± 0.01^{b}	Brownish white	Patchy	Spongy
(J)	86.67 ± 3.33^{a}	1.147 ± 0.14^{b}	0.07333 ± 0.003 ^{bc}	Brownish white	Patchy	Spongy

Each value represents the mean ± SE. Means that do not share the same letter are significantly different. Results were obtained after 21 days of the third subculture. (A) 0.5 mg L^{-1} BAP + 2.0 mg L^{-1} NAA; (B) 1.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA; (C) 1.0 mg L^{-1} BAP + 2.0 mg L^{-1} NAA; (D) 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA; (E) 2.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA; (F) 3.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA; (G) 3.0 mg L^{-1} BAP + 2.0 mg L^{-1} NAA; (H) 1.0 mg L^{-1} BAP + 0.25 mg L^{-1} 2,4-D; (I) 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} 2,4-D; (J) 1.0 mg L^{-1} BAP + 1.0 mg L^{-1} 2,4-D

Table 3: Effect of different doses of YE as a biotic elicitor and incubation period on callus culture of *T. decussatus*.

YE conc. (mgl^{-1})	Harvesting time after 15 days		Harvesting time after 30days	
	Mean fresh weight (g) ± SE	Mean dry weight (g) ± SE	Mean fresh weight (g) ± SE	Mean dry weight (g) ± SE
0 (control)	1.687 ± 0.038[d]	0.167 ± 0.012[b]	5.217 ± 0.124[c]	0.23 ± 0.006[c]
50	4.02 ± 0.053[a]	0.223 ± 0.007[a]	7.78 ± 0.128[b]	0.283 ± 0.003[b]
100	3.51 ± 0.067[b]	0.213 ± 0.003[a]	5.41 ± 0.232[c]	0.207 ± 0.003[cd]
150	2.343 ± 0.087[c]	0.117 ± 0.009[c]	10.72 ± 0.201[a]	0.337 ± 0.003[a]
200	1.73 ± 0.032[d]	0.073 ± 0.003[de]	3.787 ± 0.13[d]	0.187 ± 0.003[d]
250	1.696 ± 0.043[d]	0.083 ± 0.003[d]	3.817 ± 0.162[d]	0.11 ± 0.006[e]
300	1.483 ± 0.018[d]	0.043 ± 0.003[e]	3.607 ± 0.096[d]	0.08 ± 0.006[f]

Each value represents the mean ± SE. Means that do not share the same letter are significantly different. The initial callus weight for all treatments was 1.0 g.

Table 4: Effect of different doses of SA as an abiotic elicitor and incubation period on callus culture of *T. decussatus*

SA conc. (µM)	Harvesting time after 15 days		Harvesting time after 30days	
	Mean fresh weight (g) ± SE	Mean dry weight (g) ± SE	Mean fresh weight (g) ± SE	Mean dry weight (g) ± SE
0 (control)	1.686 ± 0.037[cd]	0.166 ± 0.012[abc]	7.78 ± 0.128[b]	0.336 ± 0.003[b]
50	1.8 ± 0.044[bc]	0.166 ± 0.009[abc]	4.367 ± 0.766[c]	0.216 ± 0.009[c]
100	1.833 ± 0.049[bc]	0.163 ± 0.007[bc]	5.487 ± 0.221[bc]	0.32 ± 0.006[b]
150	2.03 ± 0.061[b]	0.203 ± 0.012[ab]	7.473 ± 0.058[bc]	0.33 ± 0.012[b]
200	2.563 ± 0.041[a]	0.193 ± 0.009[ab]	8.73 ± 0.856[b]	0.336± 0.018[b]
250	2.63 ± 0.068[a]	0.216 ± 0.018[a]	15.01 ± 1.02[a]	0.47 ± 0.031[a]
300	1.483[d] ± 0.027[d]	0.14 ± 0.006[c]	6.2 ± 1.03[bc]	0.233 ± 0.009[c]

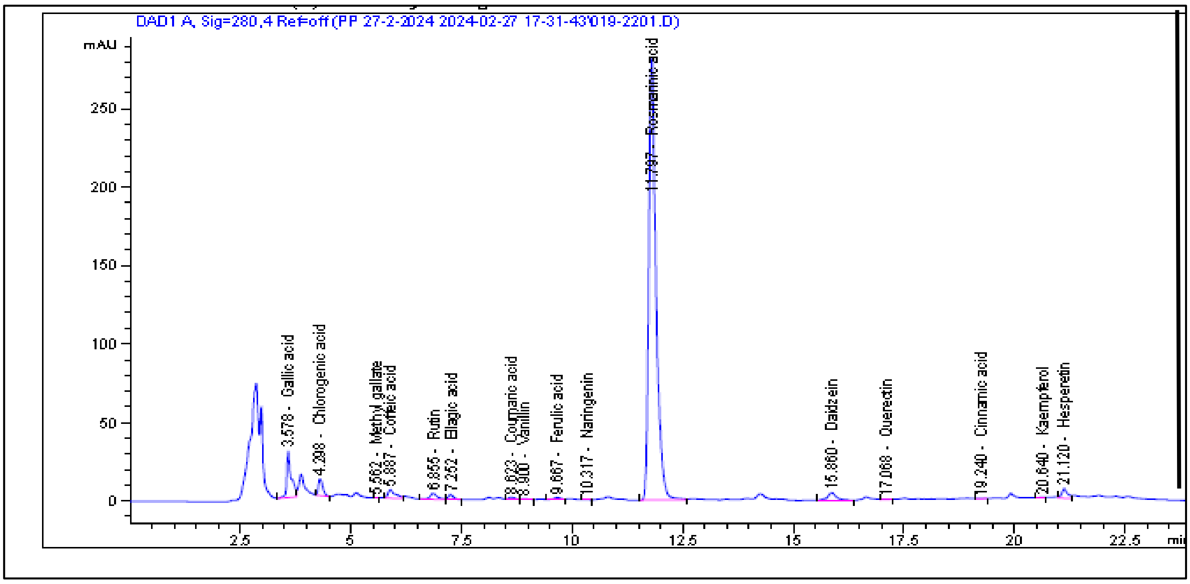
Each value represents the mean ± SE. Means that do not share a letter are significantly different. The initial callus weight for all treatments was 1.0 g.

Table 5: Effect of biotic elicitor (YE) on the concentration of active constituents in callus of *T. decussatus*

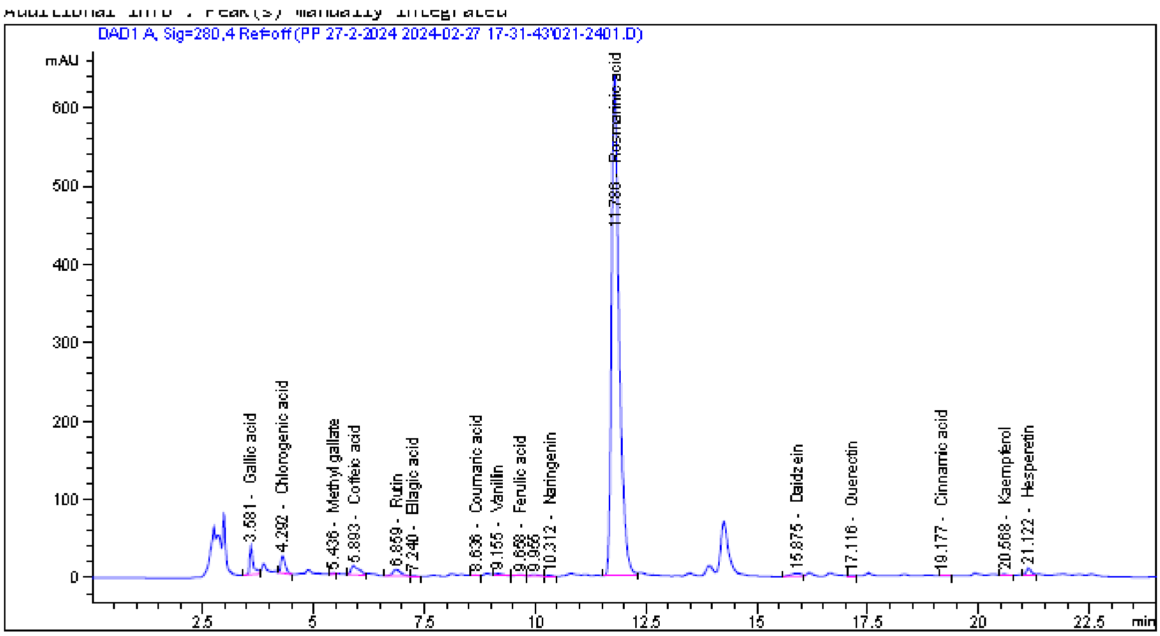
	Mother plant			Control			Biotic elicitation (YE)		
Active constituents	**Area**	**Conc. (μgml^{-1})**	**Conc. (μg g^{-1})**	**Area**	**Conc. (μgml^{-1})**	**Conc. (μg g^{-1})**	**Area**	**Conc. (μgml^{-1})**	**Conc. (μg g^{-1})**
Gallic acid	386.61	33.30	666.08	153.44	13.22	264.36	202.68	17.46	349.19
Chlorogenic acid	57.91	8.03	160.70	42.64	5.92	118.31	72.86	10.11	202.17
Catechin	5.83	1.39	27.79	0.00	0.00	0.00	0.00	0.00	0.00
Methyl gallate	13.88	0.76	15.12	13.00	0.71	14.16	0.95	0.05	1.03
Coffeic acid	151.42	13.09	261.90	38.64	3.34	66.84	64.15	5.55	110.95
Syringic acid	42.89	3.09	61.74	12.03	0.87	17.32	0.00	0.00	0.00
Rutin	317.20	52.15	1042.90	24.58	4.04	80.82	44.15	7.26	145.16
Ellagic acid	12.28	1.25	24.99	6.54	0.67	13.32	27.16	2.76	55.28
Coumaric acid	36.74	1.33	26.64	3.10	0.11	2.25	16.83	0.61	12.21
Vanillin	104.41	4.14	82.90	4.86	0.19	3.86	6.37	0.25	5.06
Ferulic acid	56.44	3.40	68.02	6.17	0.37	7.44	17.02	1.03	20.51
Naringenin	1.84	0.18	3.54	3.98	0.38	7.65	1.32	0.13	2.53
Rosmarinic acid	4346.79	465.78	9315.58	898.37	96.26	1925.29	3224.39	345.51	6910.18
Daidzein	19.30	1.31	26.19	9.19	0.62	12.47	63.94	4.34	86.77
Querectin	33.36	2.09	41.81	3.08	0.19	3.86	3.44	0.22	4.31
Cinnamic acid	10.61	0.20	3.98	4.45	0.08	1.67	2.29	0.04	0.86
Kaempferol	11.53	0.88	17.55	4.18	0.32	6.36	3.43	0.26	5.21
Hesperetin	78.38	3.94	78.85	31.92	1.61	32.11	56.10	2.82	56.43

Table 6: Effect of abiotic elicitor (SA) on the concentration of active constituents in callus of *T. decussatus*

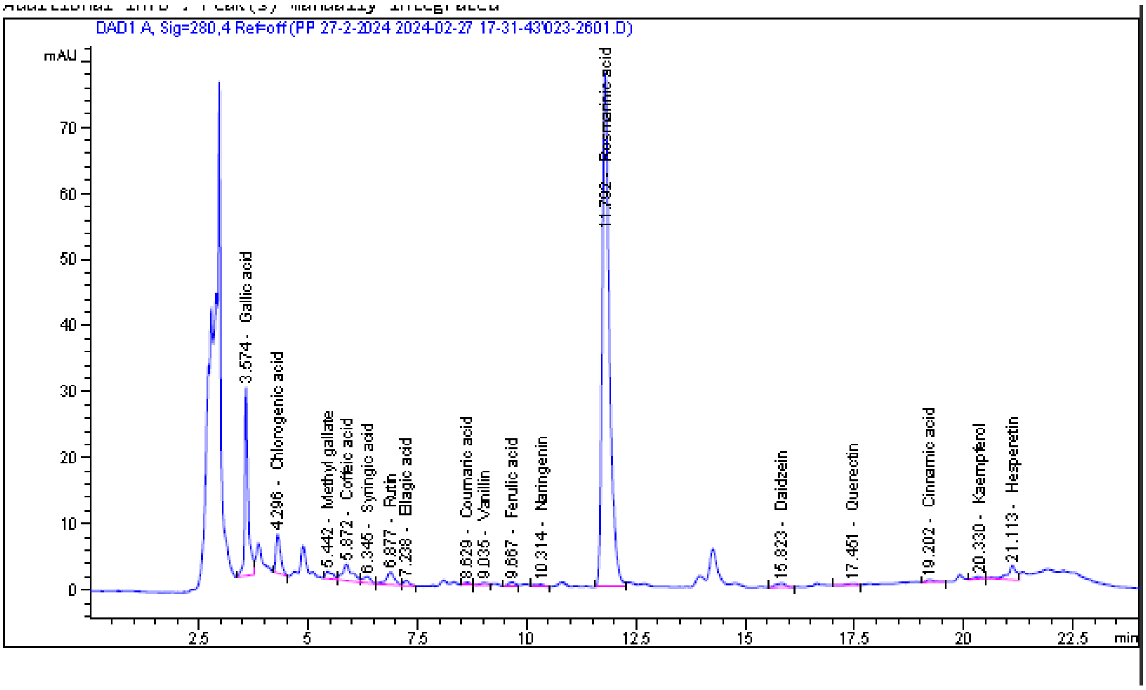
Active constituents	Mother plant			Control			Abiotic elicitation (SA)		
	Area	Conc. (μg ml^{-1})	Conc. (μg g^{-1})	Area	Conc. (μg ml^{-1})	Conc. (μg g^{-1})	Area	Conc. (μgml^{-1})	Conc. (μg g^{-1})
Gallic acid	386.61	33.30	666.08	153.44	13.22	264.36	208.63	17.97	359.43
Chlorogenic acid	57.91	8.03	160.70	42.64	5.92	118.31	152.98	21.23	424.51
Catechin	5.83	1.39	27.79	0.00	0.00	0.00	0.00	0.00	0.00
Methyl gallate	13.88	0.76	15.12	13.00	0.71	14.16	8.94	0.49	9.74
Coffeic acid	151.42	13.09	261.90	38.64	3.34	66.84	146.72	12.69	253.77
Syringic acid	42.89	3.09	61.74	12.03	0.87	17.32	0.00	0.00	0.00
Rutin	317.20	52.15	1042.90	24.58	4.04	80.82	110.12	18.10	362.08
Ellagic acid	12.28	1.25	24.99	6.54	0.67	13.32	6.27	0.64	12.75
Coumaric acid	36.74	1.33	26.64	3.10	0.11	2.25	8.20	0.30	5.94
Vanillin	104.41	4.14	82.90	4.86	0.19	3.86	37.96	1.51	30.14
Ferulic acid	56.44	3.40	68.02	6.17	0.37	7.44	14.10	0.85	17.00
Naringenin	1.84	0.18	3.54	3.98	0.38	7.65	9.98	0.96	19.19
Rosmarinic acid	4346.79	465.78	9315.58	898.37	96.26	1925.29	7612.22	815.69	16313.73
Daidzein	19.30	1.31	26.19	9.19	0.62	12.47	68.02	4.62	92.32
Querectin	33.36	2.09	41.81	3.08	0.19	3.86	4.38	0.27	5.48
Cinnamic acid	10.61	0.20	3.98	4.45	0.08	1.67	1.25	0.02	0.47
Kaempferol	11.53	0.88	17.55	4.18	0.32	6.36	13.22	1.01	20.11
Hesperetin	78.38	3.94	78.85	31.92	1.61	32.11	72.69	3.66	73.12



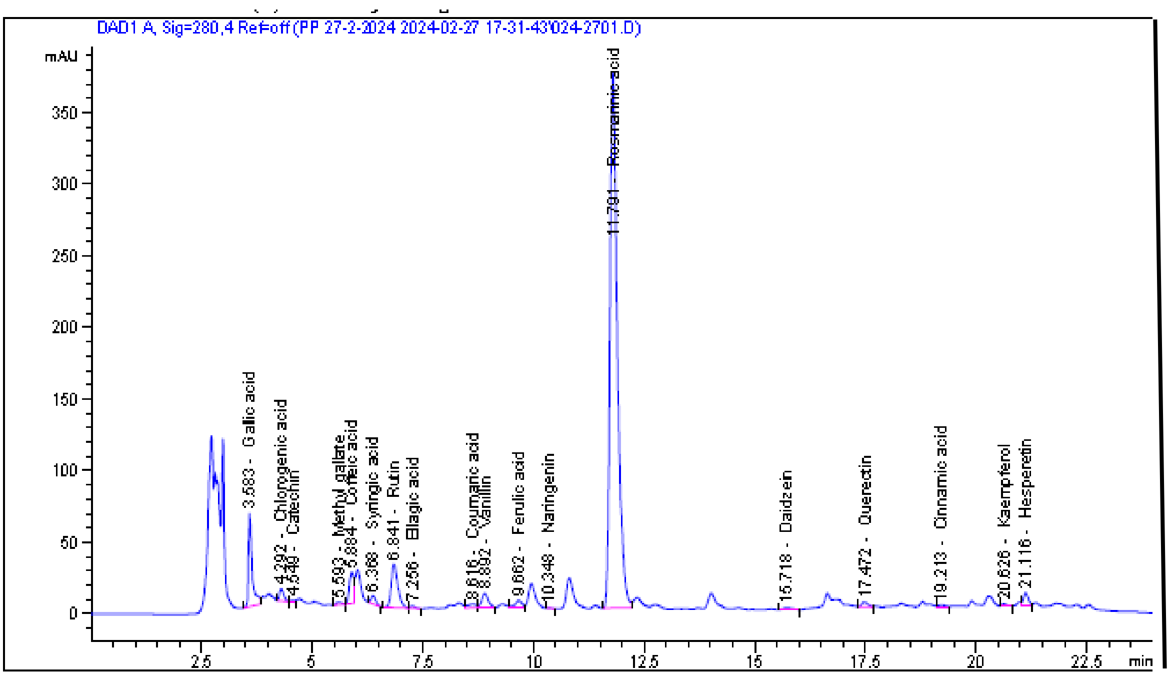
Graph 1: HPLC chromatogram showing the quantity of various phenolic compounds in callus of *T. decussatus* elicited with yeast extract.



Graph 2: HPLC chromatogram showing the quantity of various phenolic compounds in *T. decussatus* elicited with salicylic acid.



Graph 3: HPLC chromatogram showing the quantity of various phenolic compounds in callus of *T. decussatus* on control medium.



Graph 4: HPLC chromatogram showing the quantity of various phenolic compounds in mother plant of *T. decussatus*.

تحفيز تكوين الكالس وزيادة إنتاج الكتلة الحيوية والمكونات الصيدلانية لنبات الزعتران كنبات طبي مهدد بالإنقراض في مصر

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الملخص العربي:

الزعتران هو نبات طبي عشبي معمر مهدد بالإنقراض ينتمي إلى عائلة الشفويات .(*Lamiaceae*) تُعتبر أنواع الزعتر من الأعشاب الأكثر شعبية في منطقة البحر الأبيض المتوسط بسبب قيمتها الطبية والغذائية. ومع ذلك، فهي في الوقت نفسه مهددة بالإنقراض بسبب التجميع الجائر. تم إنشاء وتنفيذ بروتوكول لتحفيز تكوين الكالس في الزعتران بإستخدام بيئة موراشيجي وسكوج . تم إضافة تركيزات مختلفة من 6-benzylaminopurine (BAP) و (NAA) Naphthalene acetic acid أو2,4-dichlorophenoxyacetic acid (2,4-D). تم تحفيزتكوين الكالس بإستخدام أنواع مختلفة من الأجزاء النباتية المنفصلة من نباتات ناتجة معمليًا. تم تحقيق أعلى نسبة لتحفيز الكالس وهي 100% عن طريق زراعة أجزاء من الساق على بيئة موراشيجي وسكوج المزودة بـ 0.1 ملجم/لتر من BAP و 0.25 ملجم/لتر من 2,4-D ، مما أدى إلى الحصول على 16.72 جرام من الكالس الطازج و 0.37 جرام وزن جاف.

تمت دراسة تأثير التحفيز الحيوي (مستخلص الخميرة) وغير الحيوي (حمض الساليسيليك) على إنتاجية الكالس وتراكم المركبات الثانوية. أدى أفضل تركيز لمستخلص الخميرة (150 ميكرومول) إلى أعلى وزن طازج للكالس وهو 10.72 جرام، بينما أنتج أفضل تركيز لحمض الساليسيليك (250 ميكرومول) أعلى وزن طازج للكالس وهو 15.01 جرام بعد 30 يومًا من الزراعة. أظهر حمض الساليسيليك (SA) تفوقًا في تعزيز تطور الكالس، كما يتضح من زيادة الوزن الطازج والجاف. أعطى كلا المحفزين نتائج إيجابية في تعزيز تراكم المركبات الثانوية وزيادة كمية بعض المركبات الفينولية التي لها تأثيرات بيولوجية هامة وفوائد لصحة الإنسان

الكلمات الاسترشادية: التولد، زعتر، المستخرج، المستقبلات الثانوية، حمض السالسيليك، العائلة الشفوية، مستخلص الخميرة.