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Enhancement of the Production of Tropane Alkaloids in the *Hyoscyamus Niger* L. Callus Using Different Biotic Elicitors

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Abstract

This study looked at the amount of tropane alkaloids in both the leaves of the mother plant and the callus cultures of *Hyoscyamus niger* L. It also looked at how biotic elicitation affects the growth of callus cultures and increases the production of alkaloids. The investigation identified and quantified three primary tropane alkaloids (Hyoscyamine, Scopolamine, and Atropine) from both sources. Remarkably, the callus cultures exhibited obviously higher levels of alkaloids in comparison to the leaves of the mother plant, implying their potential as a valuable reservoir for alkaloid production. Concerning the effects of biotic elicitation, the study showed that the application of chitosan (CHT), yeast extract (YE), and fungal extract of *Trichoderma asperellum* yielded a substantial decrease in callus weight when contrasted with the control group. This decrease became more pronounced with escalating concentrations of the elicitors. In terms of alkaloid synthesis, a clear correlation between the concentration of bio stimulants and tropane alkaloid levels was established. Particularly, CHT elicitation displayed the most pronounced enhancement in levels of tropane alkaloids among the three elicitors. Notably, the highest CHT concentration of 40 mg/L yielded the most elevated levels of alkaloids, measuring at 25.3 µg/g for Hyoscyamine, 31.2 µg/g for Scopolamine, and 21.5 µg/g for Atropine. This represents approximate percentage increases in concentration of 208%, 183%, and 198%, respectively, when compared to the control treatment. Generally, these findings carry significant implications for advancing tropane alkaloid biosynthesis, with potential applications spanning the pharmaceutical and biotechnology sectors.

Keywords: Atropine**,** callus cultures, *Hyoscyamus niger* L., Hyoscyamine, Scopolamine.

تعزيز إنتاج قلويدات التروبان في كالس *Hyoscyamus niger* L. باستخدام محفزات حيوية مختلفة.

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الخلاصة

بحثت هذه الدراسة تركيز قلويدات التروبان في كل من أوراق النبات الأم ومزارع الكالس لنبات *Hyoscyamus niger* L.، ودرست أيضًا كيفية تأثير التحفيز الحيوي على نمو مزارع الكالس و زيادة إنتاج القلويدات. شخصت الفحوصات كمية ثلاثة قلويدات التروبان الأولية (هيوسيامين، سكوبولامين، والأتروبين) من كلا المصدرين. ومن اللافت للنظر أن مزارع الكالس أظهرت مستويات أعلى بشكل واضح من القلويدات مقارنة بأوراق النبات الأم، مما يدل على إمكاناتها كمستودع مهم لإنتاج القلويدات. وفيما يتعلق بآثار التحفيز الحيوي، أظهرت الدراسة أن تطبيق كايتوسان (CHT)، ومستخلص الخميرة (YE)، والمستخلص الفطري *Trichoderma asperellum* أدى إلى انخفاض في وزن الكالس عند مقارنته مع مجموعة السيطرة، أصبح هذا الانخفاض أكثر وضوحًا مع تصاعد تركيزات المحفزات. فيما يتعلق بتخليق القلويد، تم إنشاء علاقة واضحة بين تركيز المنشطات الحيوية ومستويات قلويدات التروبان. على وجه الخصوص، أظهر المحفز CHT التحفيز الأكثر وضوحًا في مستويات قلويدات التروبان بين المحفزات الثلاثة. ومن الجدير بالذكر أن أعلى تركيز لـ CHT 40 mg/L أنتج أعلى مستويات القلويدات، حيث بلغ 25.3 µg/g للهيوسيامين، و 31.2 µg/g للسكوبولامين و 21.5 µg/g للأتروبين. وهذا يمثل زيادة تقريبية في التركيز (208%، 183%، 198%)، على التوالي، بالمقارنة مع معاملة السيطرة. بشكل عام تحمل هذه النتائج آثارًا كبيرة على تقدم عملية التخليق الحيوي لقلويدات التروبان، مع تطبيقات محتملة تشمل قطاعات الأدوية والتكنولوجيا الحيوية

1. Introduction

Tropane alkaloids are found in plants of the Solanaceae family. Hyoscyamine and Atropine exhibit comparable mechanisms of action and effects to those of scopolamine. Scopolamine is the third natural chemical compound found in the parts of the datura plant, which is also known as hyoscine [1&2]. Atropine and hyoscyamine are two closely related tropane alkaloids that have similar effects on the body. Atropine is a more stable and versatile tropane alkaloid than hyoscyamine. It is also more effective in treating bradycardia and tachycardia, which makes it an important drug in resuscitation procedures [3]. Atropine in eye drops is also used to relax the eye muscles and treat early amblyopia [4]. Other pharmacological effects of tropane alkaloids have been detected, like influences on the central nervous system, relaxant properties on smooth muscle, and antitumor activity [5]. Nevertheless, excessive concentrations of tropane alkaloids can elicit toxicity and lead to tropane alkaloid poisoning. Therefore, the utilization of plants containing these alkaloids, such as black henbane (*Hyoscyamus niger*), necessitates prudent caution and should only be undertaken under the supervision and guidance of a healthcare expert [6]. Tropinone serves as the pivotal intermediary compound within the tropane alkaloid biosynthetic pathway. Previous work [7] demonstrated that two reductases play an essential role in the synthesis of active compounds by sending tropinone down different pathways. In order to synthesize hyoscyamine, l-hyoscyamine is first hydroxylated to make 6-hydroxy hyoscyamine. Then, the 6-hydroxy derivative is epoxidized [1]. On the other hand, scopolamine is made when enzymes use a series of biochemical reactions to turn hyoscyamine into scopolamine [8]. Researchers have been actively exploring methodologies to amplify the synthesis of these alkaloids within the plant, focusing particularly on *in vitro* cultures. Elicitation is a process that can be used to stimulate plant defense mechanisms and increase the production of phytochemicals [9]. Biotic elicitors, such as chitosan, yeast extracts, and fungal extracts, represent compounds capable of instigating the plant to generate secondary metabolites, including tropane alkaloids [10]. Chitosan (CHT), a carbohydrate derived from chitin (found in the cell walls of fungi and yeast), is formed under alkaline conditions through partial deacetylation or enzymatic hydrolysis via chitin deacetylases [11], as well as synthesis from

crab shells [12]. Several investigations have indicated that CHT has the potential to stimulate metabolic pathways associated with the synthesis of phenolic compounds, which serve as precursors for tropane alkaloids [13]. Additionally, biotic elicitors, such as yeast extracts (YE), have been successfully used within different *in vitro* cultures of Solanaceae plants. Hong *et al.* [14] found that the production of scopolamine in *H. niger* root cultures went up when the amount of YE was between 0.5 and 1 g/L, while Hedayati, and Nourozi [15] found that adding YE at a concentration of 5.46 M to a hairy root sped up the production of atropine and scopolamine by a large amount in *Atropa belladonna*. Also, fungal extracts (FE) are used in different cultures to increase new and different SM [16]. For example, in *C. roseus* cell cultures, the investigators revealed that a 1% concentration of FEs from *Priformospora* and *Trichoderma* exhibited significant efficacy in generating elevated vinblastine levels, in contrast to the control [17]. In a recent study, calli of *Aquilaria malaccensis* treated with extracts of *F. solani* exhibited heightened production of fatty acid derivatives, as tested through gas chromatography-mass spectrometry (GC-MS) [18].The primary focus of this study was to explore the tropane alkaloid content in both mother plant leaves and callus cultures of *H. niger*. The specific objectives encompassed the determination of optimal concentrations for CHT, YE, and *Trichoderma asperellum*, aiming to maximize alkaloids for the callus.

2. Materials and Methods

2.1. Plant Materials and Callus Cultures

The fresh leaves of *H. niger* were collected in spring 2023 from the botanical garden situated in the College of Agricultural Engineering Sciences at the University of Baghdad. The method for preparing the leaves and initiating callus formation was carried out in accordance with the procedure outlined in the study by [19]. To briefly explain, leaf portions were immersed in 70% ethanol for a duration of 2 minutes, followed by a subsequent 10-minute exposure to a solution composed of 2% sodium hypochlorite along with a small quantity of tween-20. These leaves were then thoroughly rinsed using sterile double-distilled water (DDW) multiple times [20]. The sterilized leaf segments were then placed on a basal Murashige and Skoog (MS) medium [21], which was supplemented with 2 mg/L of naphthaleneacetic acid (NAA) and 0.5 mg/L of benzylaminopurine (BAP) from Sigma. The cultures were kept within the controlled environment of a growth chamber set at25 ± 1 ℃, with 16 hours of light followed by 8 hours of darkness. Friable callus was formed after two weeks of culture. The period of maintenance for the cultures continued for three months.

2.2. Elicitation with Chitosan

Chitosan of low molecular weight (5000) Mn was procured from Sigma-Aldrich, USA. The initial solution was prepared by dissolving it in 0.1M acetic acid. The mixture was continuously stirred at a temperature of 60 ºC until complete dissolution was achieved, and the pH was adjusted to 5.6 via HCl and NaOH [22]. Varied concentrations of CHT were made (0, 10, 20, 30, 40 mg/L) and subjected to MS medium.

2.3. Elicitation with Yeast Extract

A stock solution of yeast extract (YE; Fluka) was prepared through dissolution in DDW. concentrations (0, 25, 50, 75, and 100 mg/L) were incorporated into the culture medium, followed by pH adjustment to 5.6 and subsequent autoclaving [23].

2.4. Elicitation with Fungal Extract (FE)

The fungal culture *T. asperellum* was obtained from the Faculty of Science and Technology, University Kebangsaan, Malaysia. The procedure for preparing the fungal elicitor was executed based on the method outlined in [17]. The fungal species were

maintained on potato dextrose agar (PDA) for a period of 15 days at 28 ºC, followed by transfer to potato dextrose broth (PDB) under similar conditions with continuous agitation at 120 rpm (Stuart, UK). The crude elicitor was generated from 10-day-old cultures through filtration and subsequent washing of fungal cell walls with DDW using a Buchner funnel and Whatman (No. 1) filter paper. The fungal cells then underwent a drying process for 24 hours at 65°C. Then the cells were pulverized and suspended in a ratio of 100 g/l of DDW. Series concentrations of FE were introduced into the MS growth medium (0, 1, 2, 3, and 4 mg/L).

The sterilization by Autoclave at 121 ℃ for 20 min. was achieved for all biotic elicitors' treatments.

2.5. Measurement of Fresh and Dry Weights

After the callus was subcultured and went through the maintenance stage, it was cut into 150 mg pieces and planted on growth medium that had the same mix of auxin and cytokinin along with different kinds and amounts of biotic stimulants. All treatments were incubated under the same conditions (25 ± 1 ℃, 16 hrs. of light, and 8 hrs. of darkness) for 30 days. After the end of the incubation period, the callus was dried from moisture with filter paper and then weighed on a sensitive balance to record the fresh weight data. Samples were dried in the oven at 45 ℃ for 24 hours to record dry weight data [24].

2.6. Extraction of Tropane Alkaloids

A mass of 150 mg of callus and 2 g of leaves obtained from the field-cultivated mother plant were utilized as fresh plant material. Liquid nitrogen was used to break up the plant matter which was then treated with a mixture of MeOH, NH_4OH, and $CHCl_3$, following the steps stated in [25]. Subsequently, all extraction samples were passed through a Millipore filter with a pore size of 0.45 µm. For the determination of alkaloids, 200 µL of each sample was injected into the HPLC system.

2.7. HPLC conditions

A German SYKAM- High-performance liquid chromatography (HPLC) system with a reversed-phase ODS-C18 column (25 cm x 4.6 mm I.D.) and a UV detector was used to do HPLC analysis. The elution process was observed at 254 nm. To attain optimal separation and sensitivity, an isocratic elution method involving a blend of methanol and distilled water (80:20) ml was chosen, and it operated at a flow rate of 1.0 ml/min. Tropane alkaloids, namely Hyoscyamine, Scopolamine, and Atropine, were procured from Sigma-Aldrich.

2.8. Experimental Design and Statistical Analysis

The current study followed a completely randomized design (CRD) for its experimental setup. Tissue culture experiments comprised 10 replicates. Statistical analysis was carried out using Analysis of Variance (ANOVA) along with the Duncan test, with significance considered at a confidence level of $P \leq 0.05$. The statistical procedures were executed using IBM SPSS Statistics Software, version
26.

3. Results and Discussion

3.1. Callus induction

Callus cultures induction was achieved by employing MS medium supplemented with 2 mg/L of NAA and 0.5 mg/L of BAP. The initiation of callus growth was observed approximately two weeks after introducing leaf explants to the medium. The generated callus exhibited substantial size, was friable in texture, and displayed a white-yellowish color. The average weight of the formed callus was 950 mg. Maintenance and regular subculturing were

carried out every four weeks over a three-month period. Subsequent to maintaining the callus, an investigation was undertaken to evaluate the impact of various bio-stimulants at varying concentrations on the production of active substances and the biomass of the callus.

3.2. Content of Tropane Alkaloids in Callus Cultures and Leaves of the Mother Plant

In the current study, HPLC was used to find and measure hyoscyamine, scopolamine, and atropine in the leaf tissue of mother plants and callus cultures of *H. niger*. This research explores the variations in alkaloid levels within both. The findings unveil intriguing disparities, illuminating callus cultures' potential value in enhancing alkaloid production. The data below summarizes the alkaloid content (measured in µg/g) in mother plant leaves and callus cultures, highlighting substantial differences. Notably, callus cultures exhibit significantly higher levels of Hyoscyamine, Scopolamine, and Atropine than mother plant leaves (Figure 1). Our results agreed with other researchers who conducted that the concentration of active substances produced in the field-grown plant is lower than that in callus [26 ,27&28].

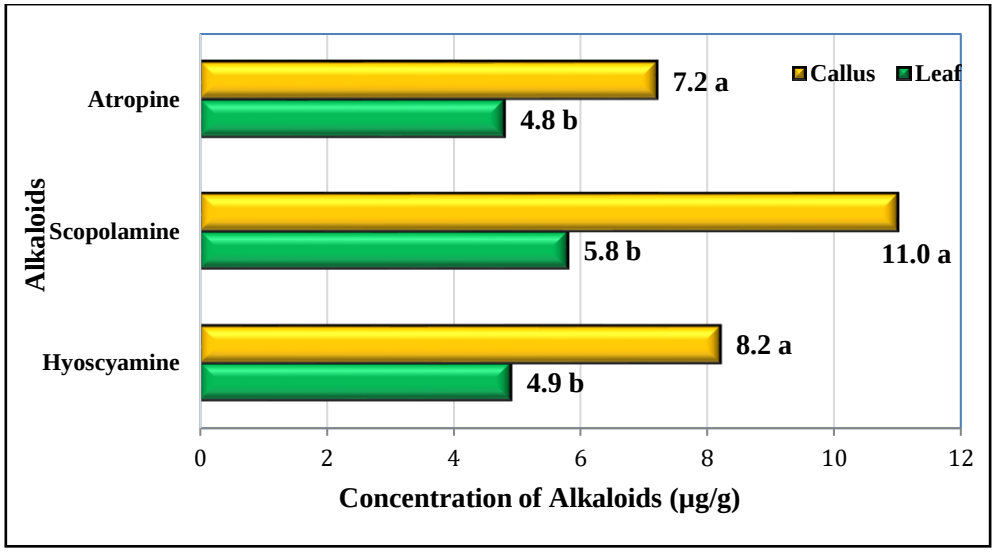

Figure 1: Comparative analysis of alkaloid content (µg/g) in mother plant leaves and callus cultures. Different letters (a–b) denote significant differences within each alkaloid.

This study specifically delves into the effects of varying concentrations of CHT, YE, and FE compounds on callus weight, offering valuable insights into their roles in optimizing callus culture. Table (1) illustrates that all concentrations of biotic stimulants utilized resulted in a significant reduction in both the fresh and dry weights of callus when compared to the control group, which exhibited the highest mean weights (488.5 mg and 45.3 mg, respectively). Notably, callus growth has an inverse relationship with rising elicitor concentrations, with growth significantly declining as biotic concentrations increased (Figure 2). This is due to abiotic elicitors were overly prevalent and could cause the generation of reactive oxygen species (ROS) in callus cells, which may result in cellular damage and directly trigger premature programmed cell death [29]. Increased concentrations of stimuli, as a result of the osmotic pressure, affect the growth of cells [30,31]. This might elucidate the notable growth reduction observed in cultures exposed to high concentrations of stimuli in our present experimentation.

Table 1: Effects of biotic elicitors on the average fresh weights (FW) and dry weights (DW) (mg) of callus cultures after 30 days of elicitation

Callus weights (mg)	Chitosan (mg/L)				
	0	10	20	30	40
FW	488.5 [a]	315.7 [b]	277.6 [c]	210.1 [d]	175.2 [e]
DW	45.3 [a]	29.1 [b]	25.4 [c]	19.3 [d]	15.1 [e]
	Yeast extract (mg/L)				
	0	25	50	75	100
FW	488.5 [a]	333.5 [b]	261.7 [c]	188.5 [d]	181.4[d]
DW	45.3 [a]	30.3 [b]	25.2 [c]	16.3 [d]	15.7 [d]
	Fungal extract (mg/L)				
	0	1	2	3	4
FW	488.5 [a]	289.9 [b]	250.1 [c]	210.6 [d]	179.0 [e]
DW	45.3 [a]	28.7 [b]	23.0 [c]	18.4 [d]	14.6 [e]

Different letters (a-e) denote significant differences between concentrations within each row.

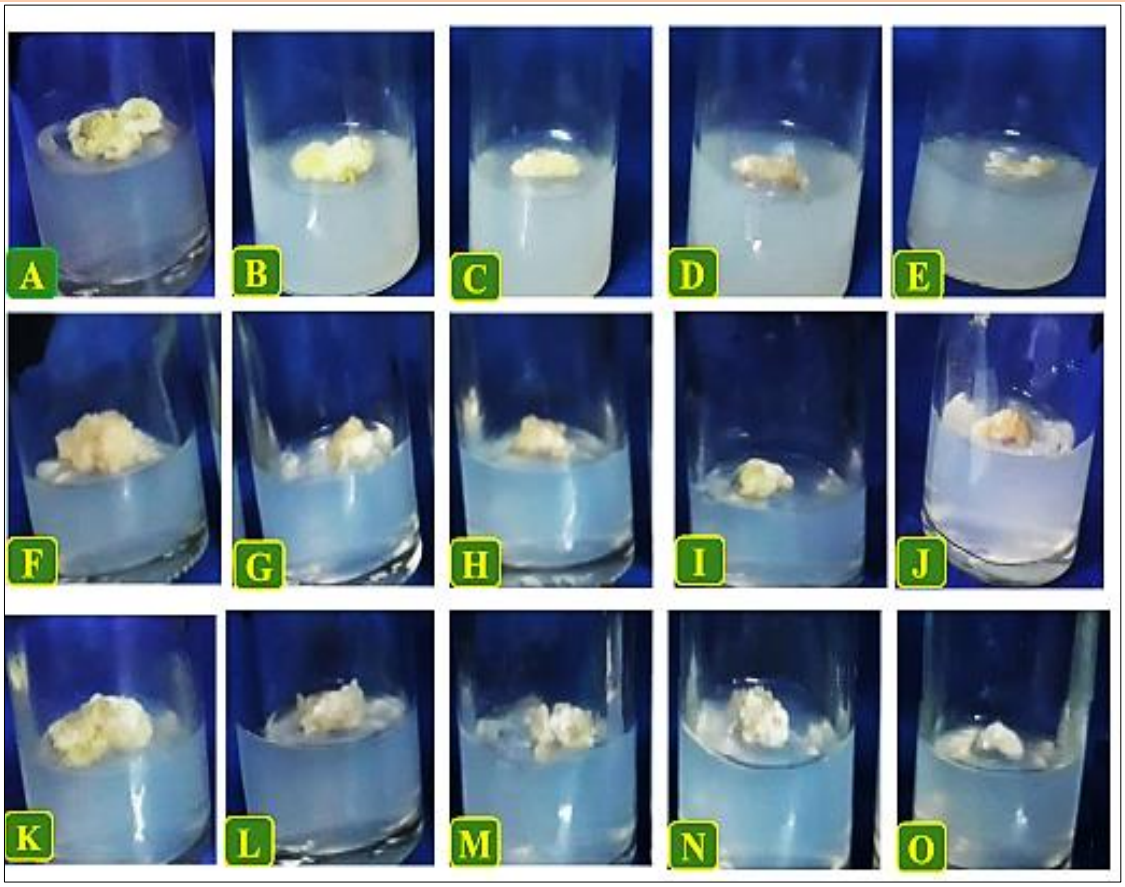

Figure 2: Graphic presentation of *H. niger* callus cultures illustrating callus morphology developed on MS (had 2 NAA and 0.5 BAP mg/L), with biotic elicitors (mg/L):
A-E: featuring chitosan treatments; (A) control, (B) 10, (C) 20, (D) 30, (E) 40.
F-J: featuring yeast extract treatments; (F) control, (G) 25, (H) 50, (I) 75, (J) 100.
K-O: featuring fungal treatments (K) control, (L) 1, (M) 2, (N) 3, and (O) 4.

3.3: Effects of Biotic Elicitation on the Content of Tropane Alkaloids in the Callus Cultures

When *H. niger* callus cultures were exposed to biotic elicitors, the amount of tropane alkaloids in the cultures kept going up. The levels of these compounds exhibited a gradual

increase in direct proportion to the concentration of the bio stimulants employed. The subsequent paragraphs provide a comprehensive breakdown of the outcomes obtained from each experimental trial.

3.3.1. *Elicitation with Chitosan*

The data presented in Table (2) clearly demonstrates a significant and pronounced correlation between the concentration of CHT in the culture medium and the levels of tropane alkaloids. As the concentration of CHT increased, there was a noticeable and consistent rise in the content of Hyoscyamine, Scopolamine, and Atropine, which measured 25.3, 31.2, and 21.5 µg/g, respectively. The approximate percentage increases in concentration for each substance are 208%, 183, and 198%, respectively, at the highest CHT concentration (40 mg/L) (Figure3). In the absence of CHT (0 mg/L), the content of the same compounds was at its lowest levels, measuring 8.2, 11.0, and 7.2 µg/g, respectively.

Table 2: Effects of chitosan concentrations (mg/L) on the content of tropane alkaloids (µg/g)

Conce. of Chitosan (mg/L)	Conce. of Alkaloids (µg/g)		
	Hyoscyamine	Scopolamine	Atropine
0	8.2 ^{e}	11.0 ^{d}	7.2 ^{e}
10	15.2 ^{d}	18.8 ^{c}	12.7 ^{d}
20	19.3 ^{c}	23.4 ^{b}	15.7 ^{c}
30	23.5 ^{b}	29.8 ^{a}	19.8 ^{b}
40	25.3 ^{a}	31.2 ^{a}	21.5 ^{a}

Different letters (a–e) denote significant differences between concentrations within each column.

It it worth noting that chitosan's impact on plant biomass accumulation could vary based on its concentration. Nevertheless, any potential negative effect on biomass is counterbalanced by its beneficial nutritional role [32]. For example, a low concentration (0.1%) of CHT demonstrated a noteworthy growth enhancement in the shoot culture of *Ruta graveolens*, concurrently leading to increased production of coumarins and alkaloids [33]. On the contrary, in the case of *Rumex vesicarius*, CHT contributed to a decrease in the weight of callus cultures, but with a significant rise in flavonoid contents, upregulation of phenolylalanine ammonia lyase (PAL) activity, and heightened antioxidant activity [34].

3.3.2. *Elicitation with Yeast Extract*

The accumulation and production of tropane alkaloids exhibit a clear response that is dependent on the dose of yeast extract (Table 3). Notably, a more substantial increase in the accumulation and production of Hyoscyamine, Scopolamine, and Atropine is observed when the yeast extract concentration is set at 100 mg/L, measuring 20.4, 25.4, and 16.8 µg/g, respectively. Conversely, the control treatment results in the lowest production levels of alkaloids. This represents a percentage increase of 148%, 130%, and 133%, respectively, compared to the control.

Table 3: Effects of yeast extract concentrations (mg/L) on the content of tropane alkaloids (µg/g).

Conce. of yeast extract (mg/L)	Conce. of Alkaloids (µg/g)		
	Hyoscyamine	Scopolamine	Atropine
0	8.2 [d]	11.0 [e]	7.2 [d]
25	9.4 [d]	13.0 [d]	8.1 [d]
50	13.0 [c]	17.1 [c]	10.3 [c]
75	16.2 [b]	20.4 [b]	13.6 [b]
100	20.4 [a]	25.4 [a]	16.8 [a]

Different letters (a–e) denote significant differences between concentrations within each column.

The efficacy of YE has been extensively studied for its potential to enhance the production of valuable compounds through *in vitro* culture methods. For instance, in the medicinal plant *C. roseus*, the application of 1.5 g/L of YE resulted in the highest alkaloid yield in both callus and various tissue cultures [35]. Similarly, YE was observed to elevate the level of SM in cell suspensions of *Azadirachta indica*, albeit at the cost of negative effects on cell growth [36]. Scientific studies have established that YE serves as a safe stimulant or elicitor due to its rich content of nucleic acids, amino acids, proteins, B vitamins, minerals, nucleotides, and fiber [37]. Additional components, such as phenolic acids and flavonoids, present in YE can activate metabolic pathways linked to the synthesis of secondary metabolites (SM) [38], while giving the highest concentration of trans-anethole [39].

3.3.3. *Elicitation with Fungal Extract (Trichoderma asperellum)*

The information in Table 4 clearly shows a strong and noticeable link between the amount of fungal extract (FE) from *T. asperellum* in the culture medium and the amounts of tropane alkaloids. So, at 0 mg/L of FE, the levels of alkaloids were at their lowest points. Conversely, 4 mg/L recorded the highest levels of Hyoscyamine, Scopolamine, and Atropine, measuring at 21.7, 27.4, and 18.0 µg/g, respectively. This represents approximate percentage increases in concentration for each substance of 164%, 149%, and 150%, respectively.

Table 4: Effects of fungal extract (*T. asperellum*) concentrations on the content of tropane alkaloids.

Conce. of fungal extract (mg/L)	Conce. of Alkaloids (µg/g)		
	Hyoscyamine	Scopolamine	Atropine
0	8.2 [e]	11.0 [e]	7.2 [e]
1	10.5 [d]	15.3 [d]	9.1[d]
2	14.6 [c]	18.2 [c]	12.2 [c]
3	17.2 [b]	21.5 [b]	14.6 [b]
4	21.7 [a]	27.4 [a]	18.0 [a]

Different letters (a–e) denote significant differences between concentrations within each column.

A team of researchers found a stimulating influence of *T. asperellum* extract on the production of tropane alkaloids. By adding different fungal elicitors to different plant culture media [40], we were able to turn on metabolic pathways that lead to the production of SM. In an experiment involving cell suspension cultures of *Aquilaria malaccensis*, the introduction of *Trichoderma* extract led to a notable reduction in biomass fresh weight while significantly inducing the production of several agar wood compounds [41]. Moreover, when callus

cultures of *A. malaccensis* were exposed to crude mycelial extracts of *F. solani,* the resulting dry weight was less than that of the control group. However, a chromone was exclusively revealed in the callus [18]. The way plants react to pathogens or biotic triggers depends on how well they can recognize signal molecules from foreign agents. These molecules are mostly made of proteins and oligosaccharides that come from the cell wall. These molecules possess the capability to initiate a range of defensive reactions in plants, including the generation of reactive oxygen species (ROS) [42]. These ROS molecules play a dual role in plant biology: they oversee normal growth and developmental processes while also serving as signal mediators that induce actions such as the hypersensitive response to production of secondary compounds with antimicrobial properties, and other defensive behaviors such as alkaloid synthesis [43].

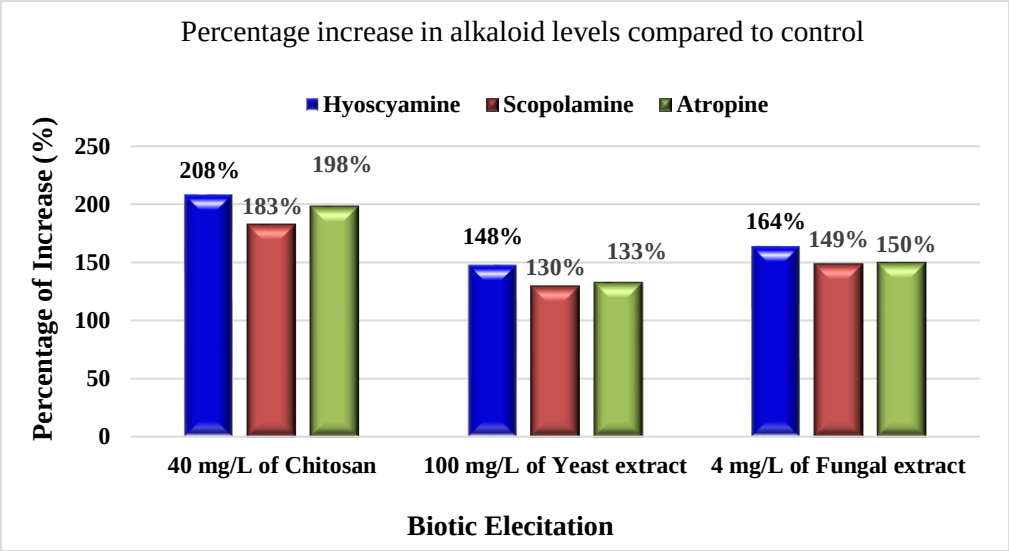

Figure 3: Percentage increase in alkaloid levels compared to the control in the callus cultures of *H. niger* treated with different biotic elicitations.

From the data on the percentage increase in alkaloid levels compared to the control for different substances (chitosan, yeast extract, and fungal extract), it is clear that chitosan had the highest percentage increase in all three alkaloids compared to the control. This suggests that chitosan had the most significant stimulatory effect on the production of these alkaloids in the given context. Hence, the use of chitosan as a supplement appears to yield the most optimal results among the options provided.

4.Conclusions

This study shows that *H. niger* callus cultures could be a good source of tropane alkaloids compared with leaves. However, concomitantly, all levels of bio stimulants displayed a favorable effect on the synthesis of the studied active compounds in the callus. The usage of chitosan as a supplement seems to be the best way to increase alkaloid production in this situation. Further research could explore the mechanisms behind these effects and optimize the elicitation process for maximum alkaloid yield.

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