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Synergistic Effect of *Fusarium oxysporum*, Silver Nanoparticles, and Abiotic stresses on Secondary Metabolite Production in *Ipomoea batatas* Callus Culture

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Abstract

This study investigates the synergistic effect between biosynthetic silver nanoparticles (AgNPs) using the fungus *Fusarium oxysporum*, and abiotic stresses (such as proline), in enhancing the production of secondary compounds within the callus culture of the sweet potato plant (*Ipomoea batatas*). The successful formation of AgNPs was confirmed by UV spectroscopy at 437 nm, and scanning electron microscope images, where particle diameters ranged between 25.5 – 50.2 nm with an average of 37.85 nm. The optimal combination to stimulate callus growth was 2 mg/L of naphthalene acetic acid (NAA) and 1 mg/L of benzyl adenine (BA), with 1.5 g of solid callus obtained in a yellowish-white color. Treatment with AgNPs at concentrations of 3 and 5 mg/L, resulted in significant increases in both soft and dry weight compared to proline treatment and control. The soft weight of the callus treated with 3 mg/L of AgNPs was about 8.60 g, and the dry weight was 2.10 g. As for the active compounds, HPLC analysis showed a significant increase in the production of flavonoids, most notably rutin, which reached 95.9 mg/L at a concentration of 5 mg/L of AgNPs, compared to 74.9 mg/L in the control and 66.0 mg/L in the intact plant. In addition, quercetin content increased to 88.7 mg/L under 5 mg/L AgNPs treatment, compared to 70.1 mg/L in the control and 59.8 mg/L in the intact plant. Similarly, kaempferol reached 80.1 mg/L under AgNPs treatment, while it was 66.8 mg/L in the control and 52.6 mg/L in the intact plant. In addition to significant increases in other compounds such as quercetin and kaempferol. An improvement in alkaloid content was also observed, reaching 2.15% in the treatment of AgNPs compared to 0.97% in the intact plant. These findings demonstrate that silver nanoparticles, which are abiotic catalysts, can effectively stimulate secondary metabolic pathways, making them a promising tool in plant tissue culture techniques to enhance the production of compounds with high therapeutic value.

Keywords: *F. oxysporum*, naphthalene acetic acid, benzyladenine, flavonoids, alkaloids

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التأثير التآزري لكل من *Fusarium oxysporum* وجزيئات الفضة النانوية والإجهادات اللاحيائية على انتاج المركبات الثانوية في مزارع الكالس لنبات *Ipomea batatas*

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الخلاصة

يستعرض هذا البحث دراسة التأثير التآزري بين الجسيمات النانوية الفضية المصنعة حيويًا (AgNPs) باستخدام الفطر *Fusarium oxysporum* والإجهادات اللاحيائية مثل البرولين، في تعزيز إنتاج المركبات الثانوية ضمن زراعة الكالس لنبات البطاطا الحلوة (*Ipomea batatas*). تم تأكيد تكوّن الجسيمات النانوية الفضية من خلال التحليل الطيفي للأشعة فوق البنفسجية عند الطول الموجي 437 نانومتر، ومن خلال صور المجهر الإلكتروني الماسح، حيث تراوحت أقطار الجسيمات بين 25.5 و 50.2 نانومتر بمتوسط قدره 37.85 نانومتر. أن التركيبة المثلى لتحفيز نمو الكالس تمثلت في استخدام 2 ملغم/لتر من حمض النفثالين أسيتيك اسد (NAA) و 1 ملغم/لتر من البنزيل أدنين (BA)، مما أدى إلى الحصول على 1.5 غرام من الكالس الصلب بلون أبيض مائل إلى الاصفرار. وعند معاملة الكالس بجسيمات AgNPs بتركيز 3 و 5 ملغم/لتر، تم تسجيل زيادات معنوية في الوزن الطري والجاف مقارنة بمعاملة البرولين والسيطرة، إذ بلغ الوزن الطري للكالس المعالج بـ 3 ملغم/لتر من AgNPs حوالي 8.60 غرام، والوزن الجاف 2.10 غرام. أما بالنسبة للمركبات الفعالة، فقد أظهرت تحاليل HPLC زيادة معنوية في إنتاج الفلافونويدات، لاسيما الروتين الذي بلغ 95.9 ملغم/لتر عند تركيز 5 ملغم/لتر من AgNPs مقارنة بـ 74.9 ملغم/لتر عند معاملة السيطرة و 66.0 ملغم/لتر في نبات الحقل. كذلك ازداد تركيز الكويرسيتين إلى 88.7 ملغم/لتر مقارنة بـ 70.1 ملغم/لتر عند معاملة السيطرة و 59.8 ملغم/لتر في نبات الحقل. بلغ الكامفيرول إلى 80.1 ملغم/لتر مقارنة بـ 66.8 ملغم/لتر عند معاملة السيطرة و 52.6 ملغم/لتر في نبات الحقل. كما سجلت زيادات معنوية في مركبات أخرى مثل الكويرسيتين والكامفيرول. وظهرت النتائج أيضاً تحسناً في محتوى القلويدات حيث بلغت نسبتها 2.15% عند المعاملة بـ AgNPs مقارنة بـ 0.97% في نبات الحقل. تشير هذه النتائج إلى أن الجسيمات النانوية الفضية بصفاتها عوامل محفزة غير حيوية يمكن أن تحفز بشكل فعال المسارات الأيضية الثانوية مما يجعلها أداة واعدة في تقنيات زراعة الأنسجة النباتية لتعزيز إنتاج المركبات ذات القيمة العلاجية العالية.

1. Introduction

Plant tissue culture is the process of cultivating plant cells, tissues, and organs on synthetic media under meticulously regulated physical conditions in a sterile environment. The emergence of plant tissue culture as an elementary science facilitates the comprehension of plant growth and development. The growth of plant cells and tissues in culture and the ability to regulate their development form the foundation for several applications in agriculture, horticulture, industrial pharmacology, and plant genetic engineering [1]. Plant tissue culture presents a cost-effective and time-saving alternative for drug development. The technique allows controlled production with virus-free plants, even those that are difficult to propagate through seed. It also aids in increasing medicinal substances and plant cell lines, demonstrating superior antioxidant activity from the culture of sweet potato tissues. Callus culture is important for creating homogeneous cell cultures that are maintained *in vitro* with nutrient media comprising vitamins, macro and micronutrients under controlled conditions [2].

Plant cells produce primary metabolites and secondary metabolites. Secondary metabolites vary among plant species in chemical nature and composition [3]. These compounds are intermediary substances or products that arise in response to stress and are important for plants to interact with their surroundings. However, they are not essential for growth and plant development [4]. Based on their chemical structure, secondary metabolites are divided into four main groups: alkaloids, terpenes, phenylpropanoids, and steroids [5]. There are some difficulties in producing secondary compounds using biochemical synthesis pathways at an industrial level because of their long and complex metabolic processes, as they have complicated structures to synthesize. Several strategies are used to increase the synthesis of these metabolites. The active transcription of genes encoding enzymes in the biosynthesis pathway regulates these secondary metabolites, resulting in the desired products [6]. An elicitor is a substance that, in living cell systems, initiates the production or release of chemicals at low concentration. Elicitation refers to the process of increasing the production of specific metabolites by adding trace amounts of elicitors. Many secondary compounds can be produced by adding elicitors to tissue culture media, such as methyl jasmonate, salicylic acid, chitosan, heavy metals, tryptophan, phenylalanine, etc. [7]. Elicitors are used to improve secondary metabolite yield by means of signal triggering [8]. The expression of many metabolite pathways can be altered by external factors, including physical and chemical environment, through the use of specific compounds (elicitors, precursors). Plants are capable of synthesizing these compounds under unfavorable conditions, and the secondary products often protect them from environmental stresses such as herbivores, heavy metals, pathogens, etc.

Elicitation refers to the mechanism through which plants enhance or promote the production of secondary metabolites, thereby ensuring their competitiveness, survival, and persistence. Thus, biotic and abiotic elicitors are utilized in plant cell cultures to enhance the formation of secondary compounds [9]. *Ipomoea batatas* L. (sweet potato) is an important crop due to its nutritional and medicinal attributes. Its capacity to thrive in diverse soil types and conditions encompasses a substantial quantity of polyphenols and other bioactive chemicals with pharmacological effects, including wound healing, anti-inflammatory, antifungal, and antibacterial activities. Sweet potatoes belong to the Convolvulaceae family and exhibit a colour spectrum that includes white, yellow, orange, and red. Nearly all components of a plant's root, leaf, and stem are consumable, although the root is the most widely consumed globally. In light of the world's escalating food crisis, academics show heightened interest in underutilized vegetables, such as sweet potato [10]. The foliage of the sweet potato plant is abundant in antioxidants and phenolic compounds [11]. An alcoholic extract of leaves contains 3.4 mg/g flavonoids, 36.5 mg/g anthocyanins, polyphenols, and 43.8 mg/g of total phenolic compounds [12]. Research highlights the effect of sweet potato extracts in type 2 diabetes, as increasing pancreatic cells through enhanced insulin leads to lowering sugar levels in the blood [13]. Accordingly, this study aims to explore novel strategies to boost the therapeutic potential of *Ipomoea batatas* by optimizing secondary metabolite biosynthesis *in vitro* using biotic and abiotic elicitors.

2. Materials and methods

2.1. Collection of the study plant

Plant leaves of *Ipomoea batatas* (L.) Lam (sweet potato), were collected from six-week-old *in vitro* rooted plantlets that had been previously cultured and acclimatized under controlled greenhouse conditions at the Department of Biology, College of Science for Women, University of Baghdad. The plant species was taxonomically identified and authenticated by Dr.Zainab Abd Awn, Assistant Professor of Plant Taxonomy in the

Department of Biology. A voucher specimen was deposited in the departmental herbarium for documentation and reference.

2.2 Explant sterilization

The plant leaf was sterilized with 0.1% HgCl_2 for 5 min after being washed with tap water for 30 to 60 min, and then was transported to petri dishes and cut into 0.5 cm long segments.

2.3 Callus production

Leaf explants (0.5 cm) were cultured on Murashige and Skoog medium supplemented with NAA and BA at concentrations of 0.0, 0.5, 1, 2, and 3 mg/l for each. For callus production, ten replications were used. Then a growth chamber was used to incubate the cultures at $25 \pm 2^\circ\text{C}$ and 16/8 h photoperiod (light/dark). After four weeks of incubation, the results were recorded for callus formation.

2.4 Isolation and preparation of the biomass of the fungus

Fusarium sp. was isolated from infected tomato roots using the protocol of Abomughaid [14]. The roots were washed thoroughly under tap water to remove soil debris and then cut into ~0.5 cm segments. These segments were surface-sterilized by immersing them in 1% sodium hypochlorite for 5 minutes, followed by five rinses with sterile distilled water to remove any residual sterilant. Sterilized pieces (four per plate) were then placed on potato dextrose agar (PDA) and incubated at $25 \pm 1^\circ\text{C}$ for 5 days. Emerging fungal colonies were examined for characteristic features. *Fusarium oxysporum* was identified based on macroscopic and microscopic features following the taxonomic key of Anama *et al.*, [15]. On PDA, colonies appeared white to pale violet with a dense, cottony texture and occasional medium pigmentation. Microscopically, the fungus produced hyaline, single-celled microconidia (oval to elliptical) in false heads on short monophialides, and fusiform, slightly curved macroconidia with three to five septa and tapered ends. Thick-walled chlamydospores were observed terminally and intercalarily. These characteristics were consistent with the description of *F. oxysporum*, and identification was further confirmed using the Vitek $\text{\textcircled{R}}$ 2 compact system (bioMérieux, France) for species-level validation. *F. oxysporum* was grown in a defined synthetic liquid medium composed of KH_2PO_4 , K_2HPO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $(\text{NH}_4)_2\text{SO}_4$, yeast extract, and glucose and incubated at $25 \pm 1^\circ\text{C}$ for 7 days with constant shaking at 150 rpm. The resulting biomass was collected by filtration (Whatman No.1) and rinsed with sterile distilled water to eliminate medium residues. A 20 g portion of the biomass was then suspended in 200 ml of sterile distilled water and incubated at room temperature for 72 hours before being filtered again for further use [16].

2.5 Preparation of elicitors

Silver nanoparticles (AgNPs) were synthesized using a biological approach involving *Fusarium oxysporum*, based on the method reported by Taha *et al.*, [17], with slight modification. Fresh fungal biomass 20 g was suspended in 200 ml of sterile distilled water and incubated at ambient temperature for 72 hours to allow the secretion of active biomolecules. The obtained filtrate was then combined with an equal volume of 1 mM AgNO_3 solution and kept in dark conditions at $28 \pm 2^\circ\text{C}$ for 48 to 72 hours. The reduction of silver ions, evidenced by a color shift to dark brown, confirmed nanoparticle formation. The AgNPs were harvested by centrifugation and preserved at 4°C for subsequent analyses. In this process, proline ($\geq 99\%$ purity) was purchased from Merck (Germany) and prepared at the desired concentration using sterile distilled water.

2.6 Examination of AgNPs elicitor solution

The presence and structure of AgNPs were confirmed using UV-Vis spectroscopy (250–550 nm range) and scanning electron microscopy (SEM), following the method by [18].

2.7 Elicitation

To enhance the biosynthesis pathway, callus was subcultured on MS medium supplemented with 1 mg/L (BA) and 2 mg/L (NAA), biotic and abiotic elicitors AgNPs as abiotic elicitor, and the abiotic elicitor (proline) was prepared at concentrations of 1, 3, and 5 mg/L for each, heated along with the medium at a temperature of 45 to 50°C. After that, the cultures were kept at 25±2°C for 16/8 (light/dark) for 30 days, and the experiment was repeated with ten replicates.

2.8 Extraction of plant material

According to Tran et al., [19], 1 g of callus from all treatments was extracted with ethanol (75%) in a (1:10) callus to ethanol ratio using an ordinary reflux apparatus at 60 °C for 8 hr. Then, the extract was filtered and dried.

2.9 Total alkaloid content qualitative estimation (Test for alkaloids)

According to previous studies [20], the total alkaloid content was determined using Dragendroff's reagent, which is prepared by mixing 0.85 g of basic bismuth nitrate and 40 ml of glacial acetic acid, then adding 20 ml of 10% potassium iodide solution. The resulting reagent was used to precipitate alkaloids from the plant extract. The test was performed by adding 1 ml of the extract to 5 ml of Dragendoff's reagent, and the formation of an orange-brown precipitate indicated the presence of alkaloids.

2.10 Determination of alkaloids

According to Sreevidya and Mehrotra [20], the quantification of total alkaloids is performed using a spectrophotometric method. After partial acid hydrolysis of the extract with 2N HCl and filtration, the solution is transferred to a separating funnel and washed with 10 ml of chloroform. The aqueous phase is adjusted to neutral PH with 0.1N NaOH. Then, 5 ml of phosphate buffer (pH 4.7) and 0.1 ml of 0.04% Bromocresol Green (BCG) solution are added, followed by extraction with 4 ml of chloroform. The resulting yellow-colored complex is measured at 470 nm using a UV-visible spectrophotometer. A standard curve is prepared using atropine sulfate in the range of 0.01-0.08 mg/ml to calculate the total alkaloid content in atropine equivalents.

2.11 Standard curve

Aliquots of atropine standard solutions (0.4, 0.6, 0.8, and 1 mg/L) were transferred to multiple separator funnels. The mixture was subsequently agitated with the extract using 1, 2, 3, and 4 ml of chloroform following the acquisition of 5 ml of BCG solution and 5 ml of pH 4.7 phosphate buffer. After collection in a 10-milliliter volumetric flask, the extracts were diluted with chloroform to modify the solution. The absorbance of the complex in chloroform was measured at 470 nm using a UV Spectrophotometer, in contrast to the blank generated earlier without atropine (SHIMADZU UV-1800).

2.12 Total amount of flavonoids

The aluminum chloride colorimetric approach was employed to ascertain the total flavonoid content of the crude plant extract, as delineated by Sultana et al., [21].

2.13 Qualitative alkaloid and flavonoid detection

The phenolic components in the crude plant extract were qualitatively analyzed using high-performance liquid chromatography (HPLC), which was performed using a SYKAM HPLC system (Germany) using a C18-OSD column (25 cm x 4.6 mm). The column's temperature was set at 30 °C. To execute the gradient elution process, eluents A (methanol) and B (1% formic acid in water (v/v)) were employed: a flow rate of 1.0 mL/min, with the first 0–5 min at 40% B, followed by 5–14 min at 50% B. Aliquots of 100 µL of samples and 100 µL of standards were injected automatically via an autosampler. The spectrum was read at 280 nm [22].

2.14 Statistical analysis

The statistical analysis system SAS (2018) program was employed to assess the effects of different factors on the studied parameters. The least significant difference (LSD) test was applied to compare means significantly, using both one-way and two-way ANOVA, as appropriate [23].

3. Result and discussion

3.1 AgNPs characterization

UV-Visible spectroscopy confirmed the successful biosynthesis of silver nanoparticles (AgNPs) by *F. oxysporum*, with a characteristic surface plasmon resonance (SPR) peak at approximately 437nm, indicative of nanoparticle formation. This result corresponds well with recent findings reporting the SPR peak in the 420-428nm range for AgNPs synthesized using *F. oxysporum* secretomes with an average particle size around 28-35nm [24], as shown in Figure 1, SEM analysis revealed nanoparticles varying in morphology, ranging from 25.5 to 50.2 nm in diameter, with a mean size of 37.85 nm.

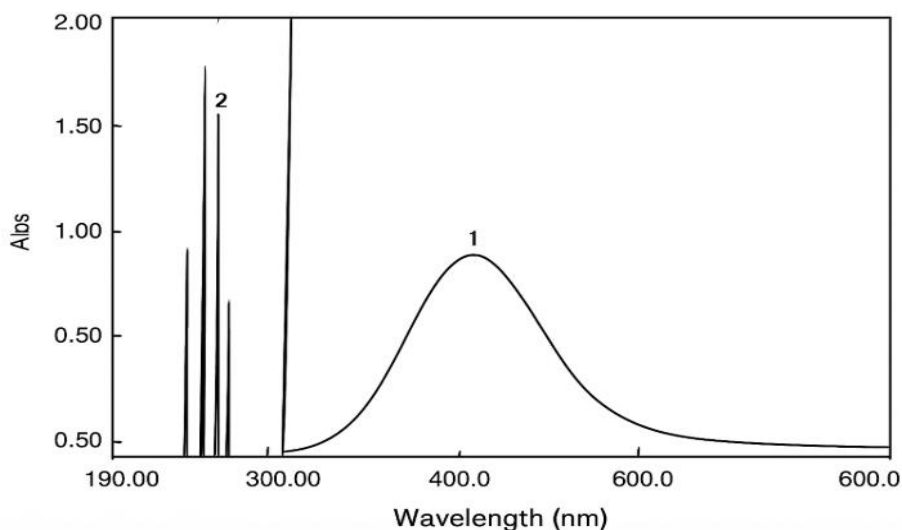


Figure 1: UV–visible spectroscopy for the AgNPs at 437 nm.

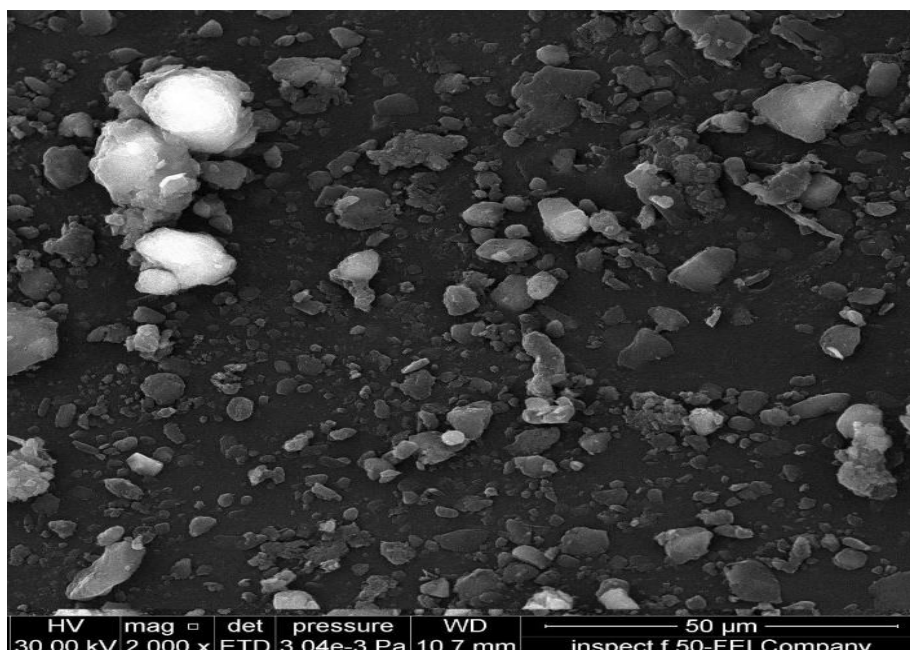


Figure 2: (AgNPs) by SFM examination.

3.2 Callus induction

The results presented in Table 1 show the effect of NAA and BA and their combination at different concentrations on callus induction on the edges of *Ipomoea batatas* leaves. The result indicated that the best combination of plant growth regulators was 2 mg/L NAA and 1 mg/L BA, which gave 1.5 g of callus from leaf explant, representing the highest weight among all concentrations that were used for callus induction and control. The combination of 2 mg/L NAA and 0.5 mg/L BA was the second-best combination, which also differed significantly from other combinations. Callus texture was compact exhibiting a white-yellowish color (Figure 3). This result may due to effect of plant growth regulators on cell division and their role on induction of functional enzymes, protein and RNA synthesis in plant cells [2] who referred that the compensation of NAA and BA can give more compact texture for callus than other types of plant hormones for callus growth on *Ipomoea batatas* leaf explants, this may explain the appearance of color and texture of *Ipomoea batatas* callus.

Table 1: Effect of varying concentrations of BA and NAA (mg/L) on the fresh weight of callus induced from leaf explants of *Ipomoea batatas* after 4 weeks of culture on MS medium.

BA(mg/l)	NAA (mg/l): Mean ±SE					Mean
	0.0	0.5	1	2	3	
0.0	0.0 ±0.0 e	0.35 ±0.07 cd	0.45 ±0.08 cd	0.50 ±0.07 c	0.30 ±0.04 cde	0.32 ±0.02 AB
0.5	0.0 ±0.0 e	0.25 ±0.04 cde	0.40 ±0.05 cd	0.86 ±0.07 b	0.37 ±0.07 cd	0.37 ±0.04 A
1	0.0 ±0.0 e	0.20 ±0.04 cde	0.0 ±0.0 e	1.5 ±0.04 a	0.42 ±0.00 cd	0.42 ±0.05 A
2	0.0 ±0.0 e	0.15 ±0.03 de	0.0 ±0.0 e	0.30 ±0.04 cde	0.20 ±0.03 cde	0.13 ±0.02 BC
3	0.0 ±0.0 e	0.0 ±0.0 e	0.0 ±0.0 e	0.0 ±0.0 e	0.0 ±0.0 e	0.00 ±0.00 C
Mean	0.00 ±0.00 C	0.19 ±0.04 BC	0.17 ±0.02 BC L.S.D. value: BA: 0.207 * , NAA: 0.207 * , BA x NAA: 0.336 * .	0.63 ±0.07 A	0.26 ±0.03 B	---

BA: benzyladenine NAA: naphthalene acetic acid ,Means having with the different big letters (main effect) and small letters (interaction) differed significantly.* ($P \leq 0.05$).

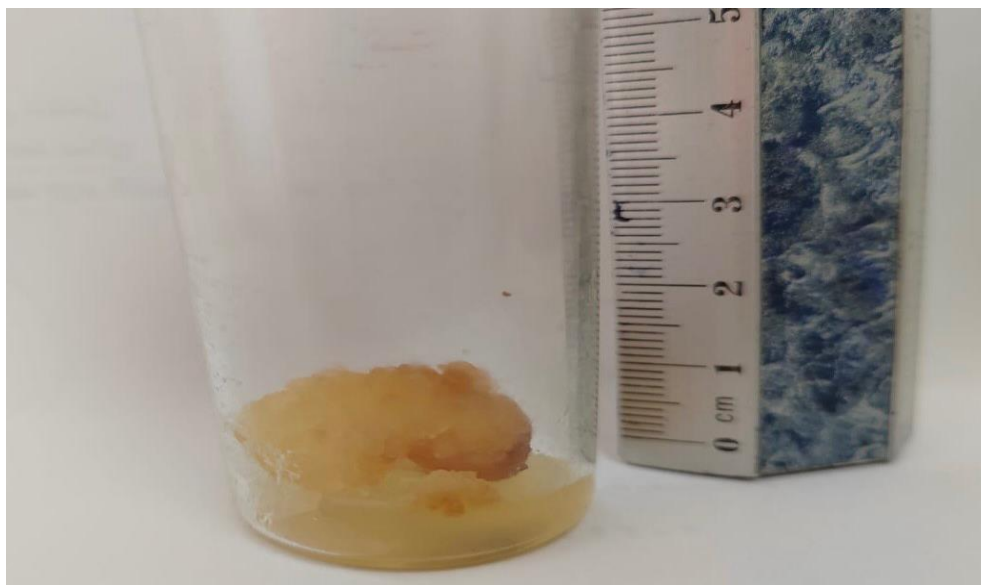


Figure 3: Callus textures exhibiting white- yellowish color induced on leaf explants.

3.3 Effect of elicitation on callus fresh and dry weight

As presented in Table 2, the application of AgNPs and proline significantly influenced the fresh and dry biomass of *Ipomoea batatas* callus. Treatment with 3 mg/L AgNPs achieved the highest biomass among single treatments, with callus fresh and dry weights of 8.60 g and 2.10 g, respectively, while proline at 5 mg/L resulted in 5.40 g and 1.30 g. These values significantly exceeded those in control and 1 mg/L treatments, though they remained comparable to the 5 mg/L AgNPs and proline treatments. This growth enhancement is likely due to the action of AgNPs as nanoelicitors that activate multiple cellular pathways, including nutrient absorption, redox signaling, and secondary metabolism, thus promoting cell division and tissue proliferation [25]. AgNPs may also interact with cell wall components and hydrolytic enzymes, facilitating cellular expansion and growth [26]. This aligns with findings in *Trigonella foenum-graecum* where AgNPs increased callus induction and secondary metabolite content, likely by modulation of hormonal and oxidative signaling [27]. Proline supplementation further enhanced biomass by acting as an osmoprotectant and antioxidant. It contributes to cellular homeostasis by stabilizing membranes and proteins and mitigating oxidative stress [28]. Notably, proline serves as a substrate for ATP production and acts structurally as an extra protein in hydroxyproline-rich glycoproteins, which reinforce cell wall architecture and support elongation [29]. The combined treatment of 3 mg/L AgNPs with 5mg/L proline produced the most substantial callus biomass, indicating a synergistic interaction. While AgNPs initiate signaling and metabolic activation, proline provides energetic and structural support to developing tissues.

Table 2: Effect of different concentrations of silver nanoparticles (AgNPs) and proline on the fresh and dry weight of callus derived from *Ipomoea batatas* leaf explants after 4 weeks of in vitro culture on MS medium supplemented with 1 mg/L BA and 2 mg/L NAA.

Vivo culture on MS medium supplemented with 1 mg/L BA and 2 mg/L NAA.				
Concentration (mg/l)			Mean $\pm$ SE	
	AgNPs		Proline	
	Fresh weight	Dry weight	Fresh weight	Dry weight
0	1.80 $\pm$ 0.17 c	0.35 $\pm$ 0.07 c	1.80 $\pm$ 0.00 c	0.35 $\pm$ 0.04 b
1	3.70 $\pm$ 0.28 bc	0.75 $\pm$ 0.13 bc	2.80 $\pm$ 0.19 bc	0.45 $\pm$ 0.13 b
3	8.60 $\pm$ 0.54 a	2.10 $\pm$ 0.00 a	5.40 $\pm$ 0.32 a	1.30 $\pm$ 0.00 a
5	6.40 $\pm$ 0.37 ab	1.20 $\pm$ 0.04 b	4.20 $\pm$ 0.27 ab	0.88 $\pm$ 0.07 b
LSD value	2.762 *	0.771 *	1.69 *	0.637 *

AgNPs: silver nanoparticles by *Fusarium oxysporum* , BA: benzyladenine , NAA: naphthalene acetic acid Means with the different letters in the same column differed significantly. * ($P \leq 0.05$).

3.4 Total alkaloids and flavonoids

Results in Table 3 indicate an increase in total flavonoids and alkaloids in *Ipomoea batatas* callus in response to elicitation by active compounds under biotic and abiotic stress conditions. This accumulation was significantly higher compared to intact plants and untreated controls after elicitation with silver nanoparticles (AgNPs) and proline. The maximum increase in total active compounds was observed at a concentration of 5 mg/L for both AgNPs and proline treatments. The role of proline as an amino acid that promotes plant growth and acts as an elicitor to enhance secondary metabolite biosynthesis has been documented [25]. Similarly, AgNPs have been reported to induce plant defense mechanisms, leading to increased production of phenols, flavonoids, and alkaloids [28]. These findings are in agreement with recent studies confirming that silver nanoparticles can act as abiotic elicitors by triggering oxidative stress responses and enhancing secondary metabolite production, including flavonoids and alkaloids, in plant tissues [30].

Table 3: Total flavonoid content (mg/100 g) and total alkaloid content (%) in callus tissues of *Ipomoea batatas* treated with proline (P) or silver nanoparticles (AgNPs), compared to control and intact plant tissues.

Treatment		ConcentrationMg/l	(Mean $\pm$ SE)	
			Total flavonoids content (mg/100gm)	Total alkaloid contents %
P	Control		52.6 $\pm$ 2.62 d	1.58 $\pm$ 0.08 ab
	Intact plant		33.25 $\pm$ 2.07 e	0.97 $\pm$ 0.03 b
		1.0	55.9 $\pm$ 2.08 d	1.96 $\pm$ 0.53 a
		3.0	63.2 $\pm$ 3.19 bc	2.30 $\pm$ 0.11 a
		5.0	66.9 $\pm$ 3.86 ab	2.48 $\pm$ 0.09 a
A		1.0	58.9 $\pm$ 2.37 cd	1.77 $\pm$ 0.08 ab
		3.0	68.4 $\pm$ 3.07 ab	1.85 $\pm$ 0.22 ab
		5.0	70.9 $\pm$ 2.44 a	2.15 $\pm$ 0.14 a
L.S.D. value		7.419 *	0.855 *	

P: Proline, A: AgNPs (silver nanoparticles by *Fusarium oxysporum*) ,means with different letters in the same column differed significantly. * ($P \leq 0.05$).

3.5 Qualitative and quantitative analysis of flavonoids

Table 4 and Figures 4 and 5 exhibit the qualitative and quantitative analysis of flavonoids under biotic and abiotic stress conditions compared with the intact plant and the control. Results showed the presence of apigenin, isorhamnetin, kaempferol, luteolin, myricetin, quercetin, and rutin after supplementation of (1, 2, and 3) mg/L of AgNPs and proline, manifesting a significant increase compared with the intact plant and control. The highest increase in active compounds was manifested in rutin under 5 mg/l AgNPs stress. This may be due to the fact that exogenous proline can increase the flavonoids in plant cells [31]. Additionally, the quantity of flavonoids in plant callus treated with proline can be increased because proline is an amino acid, which increases the soluble sugar, leading to an increase in the biosynthesis of flavonoids. This finding is compatible with the results of Arafa and Usama [32]. It has been reported that nano AgNPs can increase flavonoids since they penetrate cells, interact with DNA phosphate, and induce oxidative stress [33].

Table 4: Effect of proline (P) and silver nanoparticles (AgNPs) on the accumulation of flavonoids (mg/100 g) in *Ipomoea batatas* callus tissues compared to control and intact plant.

Flavonoids	Control	Intact Plant	1	P 3	P 5	A 1	A 3	A 5	LSD value
Apigenin	47.9 ±2.72 d	30.2 ±2.44 e	49.8 ±2.18 d	55.6 ±2.42 bcd	59.0 ±2.62 bc	51.4 ±2.41 cd	63.5 ±2.86 ab	66.9 ±3.81 a	8.37 *
Isorhamnetin	52.9 ±3.07 c	41.5 ±2.07 d	53.6 ±2.42 c	62.5 ±2.85 ab	66.5 ±2.07 a	55.9 ±2.94 bc	65.9 ±3.59 a	68.9 ±3.93 a	7.91 *
Kaempferol	66.8 ±3.29 c	52.6 ±2.19 d	68.9 ±3.92 c	73.6 ±2.64 abc	75.9 ±3.56 abc	70.8 ±3.67 bc	77.4 ±3.53 ab	80.1 ±3.70 a	7.83 *
Luteolin	59.8 ±2.74 c	47.5 ±2.71 d	61.5 ±3.16 c	67.9 ±2.75 bc	70.8 ±2.96 ab	64.5 ±2.75 b	76.0 ±3.18 b	80.0 ±3.63 a	10.2 5 *
Myricetin	42.9 ±2.35 de	36.4 ±2.19 e	44.70 ±2.47 d	49.0 ±2.16 bcd	53.6 ±3.52 abc	47.7 ±2.08 cd	56.9 ±3.06 ab	61.4 ±3.19 a	8.62 *
Quercetine	70.1 ±3.92 e	59.8 ±2.83 f	73.6 ±2.70 de	79.8 ±2.07 bcd	83.6 ±4.22 abc	76.9 ±3.56 cde	85.9 ±4.75 ab	88.7 ±4.76 a	7.58 *
Rutin	74.9 ±3.76 cd	66.0 ±3.05 d	75.9 ±3.45 c	81.6 ±4.67 bc	88.9 ±3.67 ab	78.9 ±4.07 c	92.6 ±4.95 a	95.9 ±4.73 a	8.33 *

P: proline, A: AgNPs (silver nanoparticles by *Fusarium oxysporum*), Means having with the different letters in same row differed significantly. * ($P \leq 0.05$).

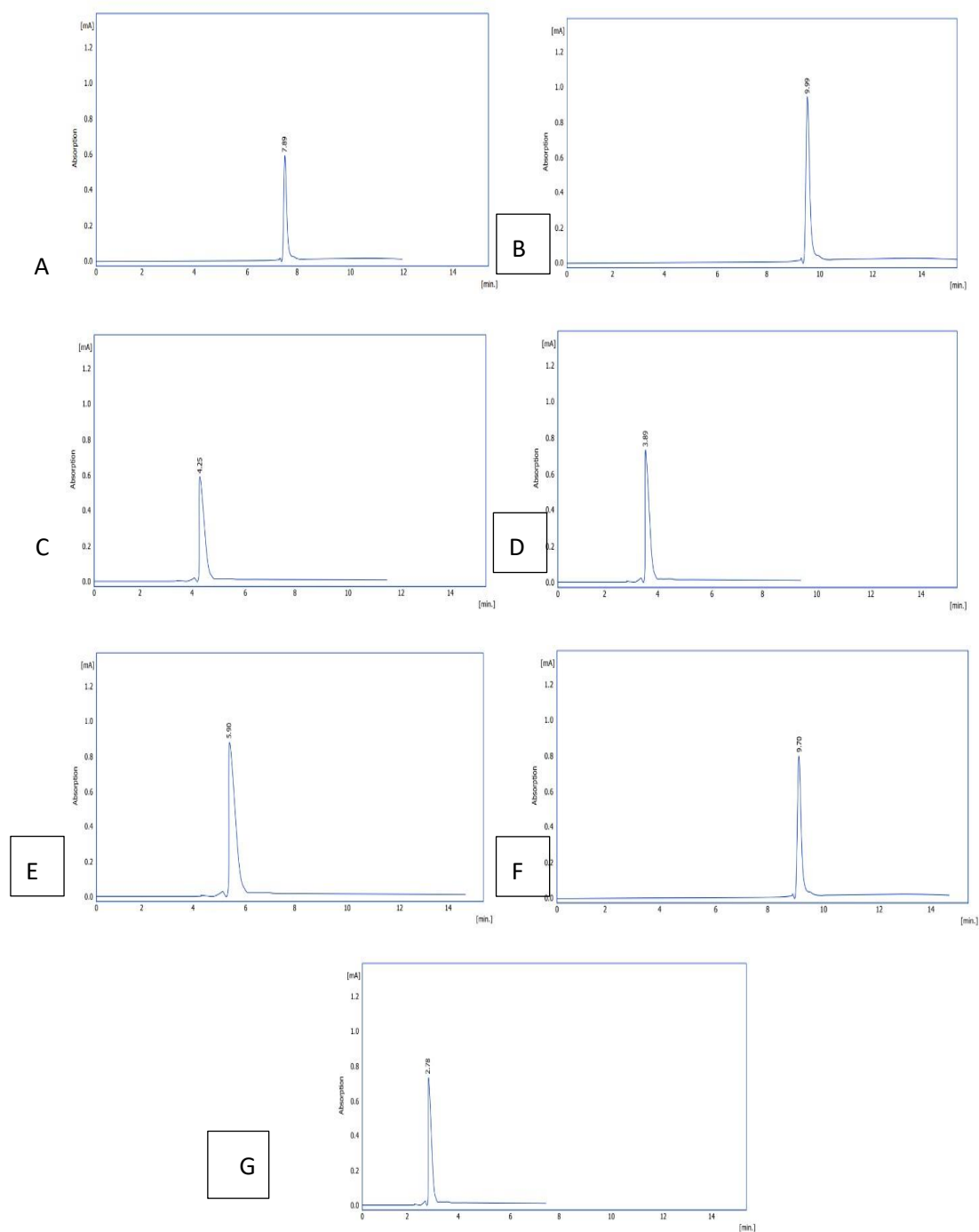


Figure 4: Standard curves for A) Apigenin, B) Isorhamnetin, C) Kaempferol, D) Luteolin, E) Myricetin, F) Quercetin, G) Rutin.

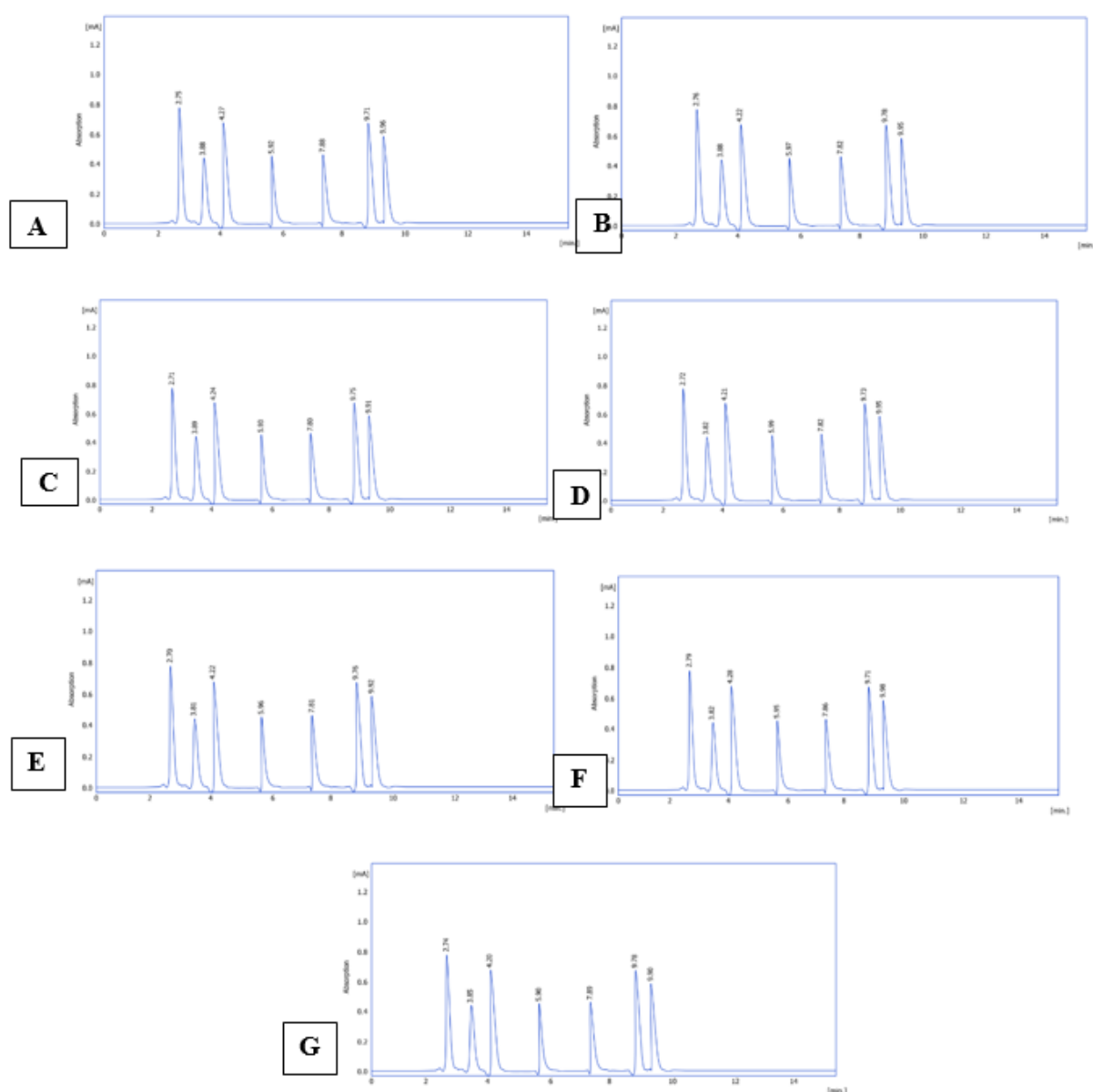


Figure 5: HPLC chromatogram of the flavonoid extracted from *Ipomoea batatas* after treatment with. A) AgNPs 1, B) AgNPs 3, C) AgNPs 5, D) Proline1, E) Proline3, F) Proline 5, and G) control.

4. Conclusion

The results of this study demonstrate that silver nanoparticles synthesized by *Fusarium oxysporum* can serve as effective abiotic elicitors to enhance callus growth and secondary metabolite production in *Ipomoea batatas* tissue cultures. The optimal combination of plant growth regulators (2 mg/l NAA and 1 mg/l BA) facilitated significant callus induction. Among the tested elicitors, AgNPs at 5 mg/l proved most effective, notably increasing flavonoid content, particularly rutin, to 95.9 mg/l. Compared to proline, intact plant and control, AgNPs induced higher accumulation of both fresh biomass and bioactive compounds, underscoring their potential application in plant biotechnology for the enhanced production of valuable phytochemicals.

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Conflict of interest.

The authors have not revealed any conflicts of interest.

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