Tikrit Journal for Agricultural Sciences (2024) 24 (4): 255-265
DOI: https://doi.org/10.25130/tjas.24.4.21

IRAQI Academic Scientific Journals
العراقية المجلات الأكاديمية العلمية
ISSN:1813-1646 (Print); 2664-0597 (Online)
TJAS
Tikrit Journal for Agricultural Sciences

The Effect of Brassinolide on Active Compounds Production of Callus of *Ocimum basilicum* L. *In Vitro*

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KEY WORDS:
Callus , Brassinolide ,Growth regulators, in vitro , *Ocimum basilicum* L

Received: 03/09/2024
Revision: 20/11/2024
Proofreading: 28/11/2024
Accepted: 05/12/2024
Available online: 31/12/2024

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ABSTRACT

The experiment was carried out at the Plant Tissue Culture Laboratory of the Department of Horticulture and Landscape - College of Agriculture - Tikrit University.The experiment aimed to know the effect of Brassinolide(BRs) on the induction of callus formation of the basil plant *Ocimum basilicum* L and its estimated content of active substrates.The basil plant seeds were sowed in the solid medium Murashige and Skoog (without plant growth regulators). True leaves were taken and grown on Murashige and Skoog medium supplied with concentrations of (0.0, 0.5, 1.0, 1.5 and 2.0) mgL^{-1} of 2,4-D mixed with a concentration of 0.5 mgL^{-1} of Kin. After 4 weeks of planting, the results showed variation in callus generation between the concentrations of 2,4-D used.The concentration of 2.0 mg L^{-1} of 2,4-D mixed with 0.5 mg L^{-1} Kin gave the highest percentage of callus formation reaching 100%, the highest fresh weight amounting to 1.0325 g and the highest callus volume (+++). The results of planting callus using different concentrations of Brassinolide (0.0, 0.01, 0.1 and 0.2) mg L^{-1} showed that callus grown on Murashige and Skoog medium supplied with a concentration (0.1 mg L^{-1}) gave the highest fresh weight (**0.6664** g)and dry weight (**0.0710** g) of callus, While the concentration(0.2 mg L^{-1}) of Brassinolide gave the highest concentration of the Caffeic acid, Luteolin, Rosemaric acid, Gallic acid andFerulic acid(15.49, 11.04, 16.90, 20.24, 7.90) ppm, respectively.

تأثير البراسينولايد على انتاج المركبات الفعالة لكالس نبات الريحان.*Ocimumbasilicum* L خارج الجسم الحي

ناظم سالم غانم

قسم البستنة وهندسة الحدائق ، كلية الزراعة ، جامعة تكريت ، العراق

الخلاصة :

نُفذت التجربة في مختبر زراعة الانسجة النباتية التابع لقسم البستنة و هندسة الحدائق –كلية الزراعة– جامعة تكريت, هدفت التجربة إلى معرفة تأثير البراسينولايد في استحثاث كالس نبات الريحان.*Ocimum basilicum* L و وتقدير محتواه من المواد الفعالة. زرعت بذور نبات الريحان في الوسط Murashige and Skoog الصلب دون اضافة منظمات نمو نباتية وتم اخذ الاوراق الحقيقية وزراعتها على وسط MS مزود بتراكيز (0.0,0.5,1.0,1.5,2.0) ملغم.لتر$^{-1}$ من 2,4-D متداخلا مع تركيز 0.5 ملغم.لتر$^{-1}$ من Kin, و بعد مرور 4 أسابيع تم جمع البيانات إذ لوحظ عدم وجود اختلاف معنوي يذكر في تكون الكالس اما في بقية الصفات فقد اعطى التركيز 2.0 ملغم.لتر$^{-1}$ اكبر حجم للكالس بلغ(+++) و اعلى وزن رطب للكالس بلغ 1.0325 غم و الذي تفوق معنويا على بقية التراكيز, بعد ذلك تم معاملة الكالس الناتج من تجربة الاستحداث (1غم) بتراكيز (0.0,0.01,0.1,0.2)% من البراسينولايد و زراعته على وسط MS مزود بتركيز 2.0ملغم.لتر$^{-1}$ من 2,4-D متداخل مع 0.5 ملغم.لتر$^{-1}$ من Kin (افضل تركيز من تجربة استحداث الكالس) و بعد مرور 4 أسابيع وجد ان تراكيز البراسينولايد لم تؤثر معنويا على نسبة بقاء الكالس عند مقارنته مع عينة السيطرة , إنما اقتصر تأثيره على الوزن الرطب و الوزن الجاف للكالس, إذ تفوقت المعاملة بالتركيز 0.1 % من البراسينولايد على بقية المعاملات فقد اعطت أعلى وزن رطب بلغ (0.66643)غم و أعلى وزن جاف بلغ (0.07104)غم. بعد ذلك تم تقدير تراكيز المواد الفعالة (Caffeic acid, Luteolin ,Rosemaric acid, Gallic acid, Ferulic acid) في الكالس المعامل بتراكيز من البراسينولايد, إذ تفوق الكالس المعامل بتركيز 0.2% من البراسينولايد في احتوائه على اعلى تراكيز من المواد المذكورة و التي بلغت ppm(15.49,11.04,16.90,20.24,7.90) و على التوالي.

الكلمات المفتاحية: منظمات النمو, الكالس, براسينولايدات , خارج الجسم الحي, نبات الريحان.

INTRODUCTION

The basil plant is an aromatic plant known as a wonderful royal fragrance. It belongs to the Lamiaceae family, there are several well-known varieties of this plant, such as French basil, American basil and Egyptian basil, today this plant can be obtained from all over the world, basil leaves (because they contain many active substrates) help prevent infertility and lower blood sugar levels, as well as stimulating producing some hormones such as estrogen and it also protects the cell structure in the body, the basil plant is used as an antibiotic for many germs and bacteria, it is also known for its ability to alleviate digestive problems,

intestinal infections, vomiting, headaches, gastritis, and fever, it also eliminates insomnia, nausea, and stress(Schwab, 2019).

Plant tissue culture technology is one of the most economically successful methods for plant propagation, it is the cultivation of a plant organ, cell or tissue in a sterile nutrient medium under controlled laboratory conditions(Singh etal,2013).This technique is used in the rapid vegetative propagation of some rare varieties or varieties that have desirable characteristics, through this technique, field crops, ornamental plants, woody plants and fruits are propagated, another important application of this technique is the production of plants free of pathogens(Cruz-Cruz etal,2013). Callus is defined as a group of unspecialized parenchyma cells resulting from cell division of meristematic cells(Hartmann etal,2002).Callus tissue is usually used in propagating plants through tissue culture by creating it, growing it and then differentiating it on nutrient media supplied with a combination of auxins and cytokinins, in addition to extracting the active compounds and estimating their concentration(Al-Hadidi,2002),and (Al-Kanani,1987).

Brassinolide (BRs) is a steroid hormone similar to steroid hormones in animals(Arora etal,2008). BRs participate in a wide range of aspects of plant development and growth and can protect the plant from various environmental pressures(Kagale etal,2007). It also works to improve photosynthesis processes and activate antioxidant enzymes, not to mention its role in providing resistance to biotic conditions such as salinity and oxidative stress(Sharma,2021). Studies have shown that basil oil contains 45 active substrates, basil contains 1% of volatile oils consisting mainly of Caffeic acid, β-Cumeric acid and Rosmarinic acid, which is considered one of the most effective antioxidants, in addition to many other effective compounds such as Thymol, Eugenol, Ursolic acid and Olenolic acid, this plant also contains β-carotene, tryptophan and many other effective compounds, it also contains proteins and vitamins, which increases its nutritional value (Yahya etal,2015).

Hassan (2019) reported in *Vignara diata* L. that the treatment with a concentration of 0.04 mgL^{-1} was superior to Brassinolide in the content of most of the active substrates that were estimated. Tianyietal (2021)Obtained the best results for enhancing and regenerating Zoysiamartrella plant callus on MS medium when treated with a concentration of 2.0 mgL^{-1} of Brassinolide.This was also achieved by (Al-Jubuoori,2022) when he treated callus of *Bougainvillea* spp plant with concentrations of (0, 0.1, 0.2 and 0.3) mgL^{-1} of Brassinolides, the highest dry weight was 0.91 g of callus treated with a concentration of 0.3 mgL^{-1}, and the highest fresh weight was 1.54 g of callus treated with a concentration of 0.2 mgL^{-1} of Brassinolide .Al-Qassam ; Ghanim ,(2023). Was also able to obtain the highest percentage of active compounds that were estimated from the callus of *Mirabilis jalapa* L. grown on MS medium and supplied with a concentration of 0.01 mg L^{-1} of Brassinolides.

MATERIALS AND METHODS

The study was conducted of the Tissue Culture Laboratory of the College of Agriculture, Tikrit University from 12 Jun. 2022 to 6 Jun. 2023, to study the induction of callus from the basil leaf and increase the production of some active substrates in it. The seeds of *Ocimum basilicum* L. were obtained from one of the offices selling ornamental plant seeds in Baghdad.

Preparation of Nutritional Medium

Murashige and Skoog (1962)an Indian medium produced by HIMEDIA, was used, and some growth regulators were added to it, according to the purpose of the study. The growth regulators used were prepared in the form of stock solutions and stored in the refrigerator at 4°C in conical flask, to prepare one liter of the medium, distilled water was placed in a beaker on a hot plate magnetic stirrer , 9 g of agar-agar was added and after it had completely dissolved, added 4.43 g of MS, then add 30 g of sucrose to complete the volume to 1000 ml using a cylinder, after that add the required plant growth regulators and adjust the pH to 5.8 ± 0.1 by adding drops of NaOH - 1 standard- or drops of HCl -1 standard- using a pH meter device MS Murashige and Skoog (1962) were used as a basic media for the initiation stage without plant growth regulators PGRs and supplemented with sucrose 30 g.L. Prior gelled the medium with agar 7 g.L the pH was set up to 5.7 ± 0.1 with 1n NaOH or 1n HCl..the nutrient medium was poured into 100 ml glass bottles, adding 20 ml to each bottle, then covering the bottle nozzles with aluminum foil. The glass bottles containing the medium were autoclaved at a temperature of 121°C and a pressure of 1.04 kg/cm² for 20 minutes, after the sterilization is complete, the bottles are taken out and left in the development room until they cool and the medium solidifies, thus becoming ready for cultivation.

Conditions for plants growing

The plants for the propagation experiments were incubated in an incubator at a temperature of 25±2°C, a light intensity of 3000 lux, and a light sequence of 16 hours of light followed by 8 hours of darkness daily(prepared from white fluorescent tubes).

Surface sterilization of seeds

The basil seeds were washed with tap water for an hour to remove of any remaining dirt and dust. After that, the seeds were soaked with 70% ethyl alcohol for 30 seconds and washed with distilled water to remove traces of alcohol. Then transferred to the cultivation cabin to be sterilized with a 15% sodium hypochlorate solution NaOCl (V:V) for 10 minutes- This solution was prepared from a commercial minor solution containing 6% sodium hypochlorate- by placing the seeds in a beaker (250 ml), then adding the sterilization solution, closing the nozzle of the beaker, and stirring continuously until the end of the sterilization period, Discarding the sterilization solution and washing the seeds seeds were rinsed off with sterile distilled water for 5 minutes three times to remove the harmful effect of the sterile substance, the seeds were planted into bottles containing MS medium (free of growth regulators) and then transferred to the incubator to form tissue culture cultures for subsequent experiments. The explants for the propagation experiments were incubated in an incubator at a temperature of 25±2°C, a light intensity of 3000 lux, and a light sequence of 16 hours of light followed by 8 hours of darkness daily

Callus formation

After four weeks the true leaves were obtained, which were taken to induce callus. The edges of the leaf were cut and the middle part of it -0.5 cm- was taken to induce callus, and placed into bottles containing MS medium supplemented with different concentrations (0, 0.5, 1.0, 1.5 and 2.0) mg L^{-1} of 2,4-D mixed with a concentration of 0.5 mgL-1 of Kin. After 4 weeks,

the following characteristics were estimated: (the Percentage of callus formation (%). Callus size:- +small (0.2 – 0.5)cm, ++medium (0.6 – 0.9)cm, +++large (1.0 – 1.2) cm. and fresh weight (g).To obtain an appropriate quantity of callus for the effect evaluation Brassinolide on active substances, the experiment was repeated with the best concentration of plant growth regulators 2.0 mg L^{-1} of 2,4-D mixed with 0.5 mg L^{-1} of Kin, which got satisfied results in the previous experiment. 1.0 g of callus were placed on bottles containing MS medium supplemented with Brassinolide in concentrations of (0, 0.01, 0.1, and 0.2) mg.L^{-1} and 2.0 mg L^{-1} of 2,4-D mixed with or 0.5 mg L^{-1} of Kin after 4 weeks following features were estimated: Percentage of callus survival, fresh weight (g), dry weight (g), estimating of the concentration of active compounds using the HPLC device.

First:Ultrasonic extraction of phenolic compounds from callus:-

Crush the sample well. Take 20g of the sample and add 100 ml of chloroform, place the mixture on a vortex for 3 hours to remove lipids from the sample.Remove the chloroform layer and dry the sample at a temperature of 50°C to ensure that the chloroform is completely removed. Take 10g of the dried sample and dissolve it using a solvent ethanol: water (70:30). Perform the extraction process using an American-origin (USA) Ultrasonic bath at room temperature for one hour. The sample is then filtered, 5 ml of the sample is taken, the solvent is removed from it using a rotary evaporator at a temperature of 40°C until it becomes a dry lumpy. The dry extracts are stored in beakers at a temperature of 4°C to prevent oxidative damage until quantitative measurements of the phenolic compounds are carried out (Herborne,1973).

Second:Perform separation in HPLC device:-

The examination was conducted in the laboratories of the Ministry of Science and Technology - Department of Environment and Water according to the method presented by (Mradu etal,2012), by using a high-performance liquid chromatography device (SYKAMN HPLC), German-originEquipped with (UV detector, Chemastation, and a ZorbaxEclipase Plus). The column temperature was 30°C. The gradient elution method was performed using eluent A (methanol) and eluent B (1% formic acid in water (v/v)) as follows:

0-40 minutes 40% B, 4-10 minutes 50% B; with a flow rate of 0.7 ml/min, the volume of the injected samples was 100µL and the calibration was 100µL. This was done automatically using an automatic sampling device with a wavelength of 280nm, as the phase was used. The carrier methanol:distilled water:formic acid (70:5:25) was the separation column (25 cm * 4.6 cm C18-ODS).

The concentration of active substance was calculated based on the following equation:

$$\text{Standard Concentration} \text{Concentration of Sample} = \frac{\text{Sample Area}}{\mathit{Standard\ Area}} \times$$

The compounds were analyzed shown in Table (1)

Table (1) Active substrates that were estimated using HPLC technology in basil plant callus grown on different concentrations of brassinolides

No.	
1	Caffeic acid
2	Luteolin
3	Rosemaric acid
4	Gallic acid
5	Ferulicacic

Figures (1), (2), (3), (4), and (5) show the standard curves for these compounds

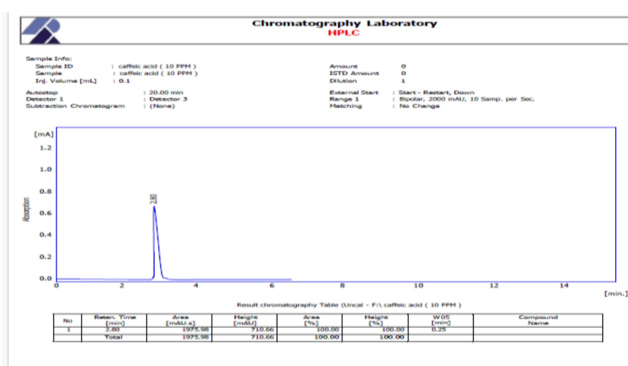

Figure (1) Caffeic acid

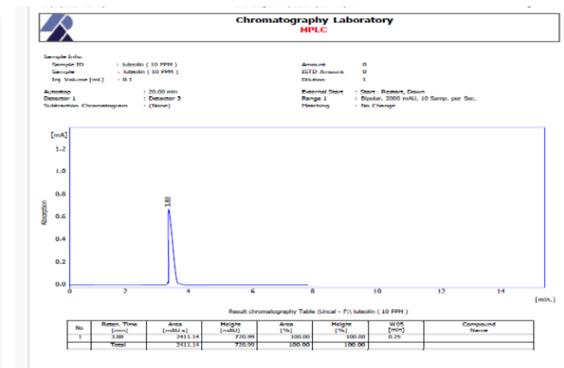

Figure (2) Luteolin

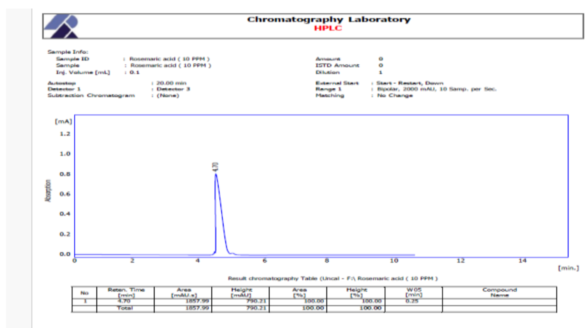

Figure (3) Rosemaric acid

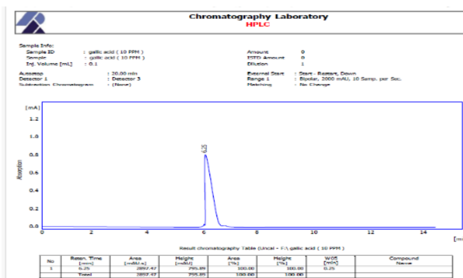

Figure (4) Gallic acid

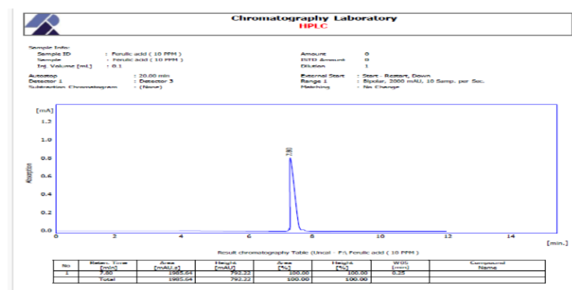

Figure (5) Ferulic acid

Experimental design and statistical analysis

The experiment design was carried out using a Completely Randomized Design (C.R.D.), with 10 replicates taken, each replicate consisting of one plant part.

The means were compared using Duncan's Multiple Range test at the probability level of 0.05, and the program SAS (2001) was used to analyze the data(Al-Rawi and Khalaf Allah,2000).

RESULTSANDDISCUSSION

The results of Table (2) show the 2,4-D treatment at concentration 2.0 mg L^{-1}gave the highest percentage of callus formation (100%), but there are no differences significantly with concentrations 0.5 and 1.0 mg L^{-1}.

On the other hand, the concentration of 2.0 mg L^{-1} increased significantly in size of the callus (+++). While the concentration of 1.5 mg L^{-1} of Brassinolid edecreased significantly (+). At the same time, the concentration of 2.0 mg L^{-1} also had the highest value in the fresh weight of the callus1.0325 g, whereas the concentration of 1.5 mg L^{-1} of Brassinolide had the lowest value 0.3043 g.

Probably due to the role of auxins in inducing callus on plant parts, and that 2,4-D is one of the auxins that encourages the formation of adventitious roots resulting from cell division and the construction of nucleic acids, adding a cytokinin with auxin to the medium leads to an increase in callus growth and encourages cell division due to the state of hormonal balance that occurs between auxin and cytokinins(Hartmann etal,2002).

It may be due to about this result that the process of callus formation depends on the potential energy of the cells and on the compatibility between the nutrient medium and the different concentrations of growth regulators, which in turn promote cell division in the plant parts, whose ability to respond varies depending on their source(Margl et al,2002).Callus induction, growth, subsequent differentiation and organ formation are accomplished by various additions to growth regulators, the presence of stimulation resulting from endogenous growth hormones or by adding growth regulators to the nutrient medium, the cells will divide, grow, the resulting plant tissue will differentiate and this explains the increase callus volume(Kassab Bashi,2020).

Table (2) effect of different concentrations of 2,4-D and concentration of 0.5 mg L^{-1} of Kin on the production of callus from basil leaves

2,4-D conc. (mgL^{-1})	Kin conc. (mgL^{-1})	Callus formation (%)	Callus volume	Fresh weight (g)
0.0		0 c	0 c	0.0 d
0.5		90 a	++ab	0.6299 b
1.0	0.5	70 ab	++ab	0.8013 a
1.5		50b	+b	0.3043 c
2.0		100 a	+++a	1.0325 a

*Different alphabets in the same column show significant difference using Duncan's Multiple Range test (P≤ 0.05) and average was calculated from ten replicates.

The effect of adding Brassinolide on some callus characteristics

Table (3) refers that there is no significant difference among different concentrations of Brassinolide in the percentage of callus survival. however,the concentration of 0.1 mg L^{-1} of Brassinolide gave significant increment in fresh and dry weights 0.6664 g and 0.0710 g, respectively. Followed by the concentration of 0.2 mg L^{-1}. By contrast the control treatment gave significant reduction in these traits 0.4873 g and 0.0537g, respectively.

The Brassinolide effects probably depended upon the ability to regulate many physiological and cellular processes that occur in plants, such as cell elongation and division, cellular synthesis of cell wall components, synthesis of various proteins, DNA and RNA distribution of manufactured materials to plant organs, nitrogen fixation and synthesis, adventitious roots, flowering, production, resistance to living and non-living stresses and other processes (Abu Zeid,2000). Brassinolide is known for its synergistic action with auxin to stimulate cell elongation (Sasse,1990).

The importance of brassinolide in the laboratory in many studies that aim to improve physiological and biochemical processes and increase the rate of regeneration by inducing callus and somatic embryos. also work to improve photosynthesis processes , activate antioxidant enzymes and most importantly brassinolide provides resistance to biotic stresses such as oxidative stress and salinity(Sharma,2021).

It is believed that brassinolide participates in the relaxation of the cell wall, which leads to elongation and cell division. This may be due to the role of brassinolide in increasing carbon metabolism, which leads to an increase in CO2 in the leaf, which represents the internal element for building carbohydrates, or perhaps due to its role in increasing internal hormones in the plants leaf such as zeatin and GA3, which stimulate growth and development. It is worth noting that increasing carbon metabolism leads to improving the characteristics of vegetative growth, such as plant height, number of leaves and fresh and dry weight of vegetative growth (Alwan,2016).

Table (3) effect of different conc. of Brassinolide on some traits of basil plant callus

BRs. conc. (mgL^{-1})	**2,4-D conc. (mgL^{-1})**	**Kin conc. (mgL^{-1})**	**Callus survival (%)**	**Fresh weight (g)**	**Dry weight (g)**
0.0			90 a	0.4873 b	0.0537 b
0.01			80 a	0.4918 b	0.0550 b
0.1	2.0	0.5	90 a	0.6664 a	0.0710 a
0.2			100 a	0.5406 ab	0.0590 ab

* Different alphabets in the same column show significant difference using Duncan's Multiple Range test (P≤ 0.05) and average was calculated from ten replicates.

Effect of different concentrationsof BRs on the content of some active substrates in the callus of a basil plant

The concentration of Brassinolide treatments had higher active substrates concentration than the control (Table 4). The concentration 0.2 % of Brassinolide recorded the highest concentration of active substrates (Caffeic acid, Luteolin ,Rosemaric acid, Gallic acid, Ferulic acid) were 15.49 , 11.04 , 16.90 , 20.24 and 7.90 ppm respectively, compared to the control treatment 12.05 , 9.25 , 14.58 , 18.25 and 6.32 ppm respectively.

The changes in the percentage of active substrates were mainly attributed to the ability of Brassinolide to stimulate cell, vascular tissue differentiation and the development of lateral roots, it may also be due to the increase in the division and elongation of parenchymal cells (Al-Khafaji,2014). The reason for the difference in concentrations and types of metabolic compounds in callus is due to the instability of its genetic material, as variation in its genetic material can occur once it remains on the medium for a long time (Muhammad and Omar,1990).Brassinolide also activates some of the genes responsible for the formation of RNA found in the cell's chromosomes, especially mRNA, thus it activates the enzymes responsible for the process of division, and elongation, which causes a change in the shape and composition of the cells (Alwan,2016).

Table (4) Effect of Brassinolides on the phenolic compounds in the callus of a basil plant

phenolic compounds (ppm)	**BRS Cocentration (mgL^{-1})**			
	0.0	**0.01**	**0.1**	**0.2**
Caffeic acid	12.05	13.25	14.00	15.49
Luteolin	9.25	9.88	10.58	11.04
Rosemaric acid	14.58	15.11	16.35	16.90
Gallic acid	18.25	19.11	19.85	20.24
Ferulic acid	6.32	6.88	7.58	7.90

CONCLUSIONS

Through this study, we conclude that adding the growth regulator 2,4-D at a concentration of 0.5 mg L^{-1} with a concentration of 0.5 mg L^{-1} of Kin to the nutrient medium gave the highest percentage of callus formation, reaching 100%, while adding the growth regulator Brassinolide at a concentration of 0.1 mg L^{-1} to the medium affected the characteristics of callus, as it gave the highest fresh and dry weight of callus, while a concentration of 0.2 mg L^{-1} of brassinolide gave the highest concentrations of all the active substrates that were estimated.

ACKNOWLEDGMENT

We would like to extend our thanks and appreciation to the staff of the Horticulture and Landscape Department, College of Agriculture, Tikrit University, for the scientific and practical support they provided to complete this research.

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