Callus generation and estimation of the apigenin content in different vegetative shoots tissues of chamomile (*Matricaria chamomilla* L.) plants

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Abstract

The results successed in callus initiation from the hypocotyl stems segments of chamomile (*Matricaria chamomilla* L.) seedlings on the solid MS (Murashige and skoog, 1962) medium supplemented with the addition of 1.0 mg L^{-1} from each of Naphthaleneacetic acid (NAA), benzyl adenine (BA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) with ratio reached to 100% after 30 days. The growth of the callus continued until it produced typical cultures and its differentiated for vegetative branches. Also, this study acheived genetic transformation of the chamomile plant with the Ri plasmid vector isolated from *Agrobacterium rhizogenes* ATCC 15834 using the direct injection technique, with superiority the concentration of 1214.32 ng μl^{-1} in the percent of hairy roots produced at 80% and vegetative branches at 94%
The results determined the apigenin content in different tissues for vegetative shoots of chamomile plant using High liquid performance chromatography (HPLC) technology, where the high concentration was 0.4451 µg ml^{-1} in the genetically transformed tissues with Ri plasmids vector at 1214.32 ng μl^{-1}, superior to the other samples and the lowest content of apigenin was in the vegetative shoots which differentiated from callus with 0.0121 µg ml^{-1}.

Keyword: *Matricaria chamomilla* L.**,** Genetic transformation, Apigenin, HPLC.

Introduction

Matricaria chamomilla L. (Asteraceae) It is a medicinal plant that has anti-inflammatory and antimicrobial properties and is also used as a sedative....etc. according to the presence of phenolic compounds [1]. M. chamomilla contains more than 120 types of medicinally active ingredients including 56 types of organic acids, 36 types of flavonoids, 28 types of terpenoids, and other compounds such as coumarins [2],The antiphlogistic activities are mainly attributed to the presence of apigenin, and its acetylated derivatives [3] Volatile secondary metabolites of plant terpenoids have important ecological and biological functions . the flavones are played an role in the overall anti-inflammatory activity, It can be mainly expressed in

the interaction between a plant and environment and the transmission of biological information [4] There are many techniques such as high performance thin layer chromatography (HPLC), thin layer chromatography (TLC), high performance liquid chromatography-ultraviolet (HPLC-UV) analyzed several hydroxycinnamic acid derivatives and flavonoids of chamomile [5] ,Apigenin is water-solubleIts, main form in which it exists of apigenin-7-O glucoside and other acylated derivatives [6] ,It has a wide range of biological and therapeutic activities due to its lipid-soluble properties, including antioxidant, anti-inflammatory, anticancer, antigenotoxic, antiallergic, neuroprotective, cardioprotective, and antimicrobial effects. These properties make apigenin an interesting substance for use in many health and food products [7].

Genetic transformation includes transferring the gene from one organism to another to create a desired variation in its genetic and biological characteristics [8]. The use of Agrobacterium rhizogenes is one of the most successful systems for plant genetic transformation, its characterized by being a negative-Gram stain, endemic to the soil, natural vector of genes to injured plant cells inclusion them within their genomes [9] This bacteria causing hairy root disease when a plant was infected [10], hairy roots can stimulate abnormal growth in a variety of plants, whether monocot or dicot, when these roots are injured or wounded, they become sensitive and may exhibit reactive responses such as increased growth or vegetative shoots production [11]; [12].

During the infection process, A. rhizogenes transfers a segment of DNA (T-DNA) to the plant genome, it's a portion of root-inducing plasmid (pRi) of [13] Formation of hairy roots initiates after the integration of T-DNA with the plant genome [14]. The use of bacteria has been successful in different types of plants, e.g, Brassica oleracea var italic [15], Soy bean [16], Raphanus sativus [17], Nigella sativa L [18], Helianthus annuus L. [19]. the study aimed to estimate the Apigenin compound quantities in different tissues of *Matricaria chamomilla* L. with High Performance Liquid Chromatography (HPLC) technique.

Materials and methods

Source the plasmids pRi-DNA of *Agrobacterium rhizogenes ATCC 15834* used in this study

The bacterial strain *A. rhizogenes ATCC 15834* was obtained from Prof. Dr. Jutta Ludwig-mueller, Technical University Dresden, Germany. pRi-DNA was isolated from bacteria grown for periods 24 and 48 hours individually using a plasmid extraction kit (Genomic, DNA Extraction Kit, korea AddPrep) [20] .The concentration and purity of isolated plasmid DNA determined used NanoDrop device at the wavelength 260-280 nm and reached 1159.24, 1214.32 ng μl^{-1} for each incubation period respectively, with values of the purity for two were 1.8.

Surface sterilization of chamomile (*Matricaria chamomilla* L.) seeds:

Chamomile seeds, *Matricaria chamomilla* L. were prepared from local markets in the city of Mosul/ Iraq, and were surface sterilized by immersing them in a 96% ethyl alcohol solution for two minutes, then immersed in a 2% sodium hypochlorite solution (NaOCI) for 5 minutes [21]. They were washed with distilled water three times per minute and placed on filter paper for drying. After that, the seeds were placed on the surface solid MS (Murashige and skoog, 1962) [22] medium. All samples were in a culture room in dark conditions at a 24 ± 2°. After seed germination with four days, they were transferred to photoperiod conditions with 16 hours of light at an illumination intensity of 2000 lux/ 8 hours dark.

Callus initiation from hypocotyl stems of chamomile (*Matricaria chamomilla* L.) seedlings and its spontaneous differentiation:

Healthy chamomile seedlings after 25 days of growth, taken from the medium, their hypocotyl stems were cutted and were placed separately in 100 ml flask containing 30 ml of solid MS medium(Murashige and skoog, 1962)supplemented with 1.0 mg L^{-1} from each of Naphthaleneacetic acid (NAA), Benzyl.adinen (BA) and 2,4-Dichlorophenoxyacetic acid (2,4-D), and the samples were placed in the culture room at above conditions. For its differentiation, the callus after formation was subcultered on the solid medium.

Transformation procedure

25-day-old of seedling pulled from MS medium, separated their hypocotyl stems with a length of 1.5 cm and their leaves have part of petiole, then inoculated using the direct injection method by sterile minute needle (Insulin Syringe) immersed its end in the 1159.24, 1214.32 ng μl^{-1} of plasmid

suspension for bacteria *Agrobacterum rhizogenes ATCC 15834* individually [23] .Stems segments were submerged at the top ends and sides, and the samples were cultures on the surface 30 mL of MS (Murashige and skoog, 1962) solid medium at a rate of 2-3 pieces/flask and were preserved in a growth incubator with a temperature of 24±2°C with 400 lux.

Preparation of standard solutions of apigenin:

The apigenin standard was dissolved in methanol to prepare a stock solution. It was prepared by dissolving the standard apigenin powder compound 0.5 mg (apigenin powder, sigma) in 1.0 ml of methanol solution, and its final concentration became 0.5 mg ml^{-1} before analysis, all samples were filtered through a 0.45µm Millipore filter. These steps ensure accurate and precise quantification of apigenin in the samples. [24].

Samples preparation

The apigenin compound was estimated from chamomile tissue extracts, which are vegetative shoots of wild plants, seedlings, differentiated from callus grown on MS (Murashige and skoog, 1962) medium containing1.0 mg L^{-1} of Naphthaleneacetic acid (NAA), Benzyl.adinen(BA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) at 30 days old and genetically transformed with Ri plasmid vector at isolated from *Agrobacterum rhizogenes ATCC 15834*,

0.5 mg of dry matter was taken from each of the above samples. they were ground individually in a ceramic mortar to obtain their fine powder and placed in flask with added 1.0 ml of 70% methanol solution, the samples were transferred into the ultrasound machine (Ultrasonic Cleaner, Germany) at a power of 50 kHz for two hours at room temperature, it was followed by filtration with a vacuum filtration device using filter paper with a hole diameter of 0.45 micrometers. The extracts were sprayed using special filters (Syringe Filter, Cellulose Acetate) with a diameter of 0.22 micrometers to obtain samples ready to be measured using HPLC [25].

Estimation of apigenin concentration by HPLC (High-Performance Liquid Chromatography):

An HPLC device that includes a double pump was used for the purpose of separating and measuring the concentration of active compounds using the mobile phase at pH 3.2. The solutions were placed before use in the sonication devices (Ultrasonic cleaners, Elmasonic s,Germany) for 10 minutes, and the examination was performed using a metal separation column.

Column specifications: Type (column C18) Column length: 250 mm, packaged material: silica granules, inner diameter: 4.6 mm. The measurement process was carried out, and the measurement process was carried out by placing 5.0 of the final extract solution for the samples in the measuring cell, each of them separately, and placed in a designated place inside the device. After obtaining the results of all samples, the retention time (RT) and area of the curve for each sample were compared with the standard apigenin sample. The concentration of apigenin in each eye was calculated using the following equation:

$$\text{Apigenin concentration} = \frac{\text{Sample curve area x standard substance concentration}}{\text{Standard substance curve area}} * \frac{\text{dilution times number}}{\text{sample weight}}$$

Results and discussion

callus initiation of chamomile (*Matricaria chamomilla* L.) and its cultures production:

The results indicate that the MS(Murashige and skoog, 1962) medium supplemented with the addition of 1.0 mg L^{-1} from each of Naphthaleneacetic acid NAA, Benzyl.adinen BA and 2,4-Dichlorophenoxyacetic acid 2,4-D were able to encourage the hypocotyl stem segments of chamomile seedlings to initiated 100% callus after 7 days, where the callus was characterized by its fragile structure and yellowish-green colour (Figure, 1.A). the induced callus cells continued their successive divisions, forming small masses after 10 days and continued to grow and produced typical cultures of callus after 30 days (Figure, 1.B).

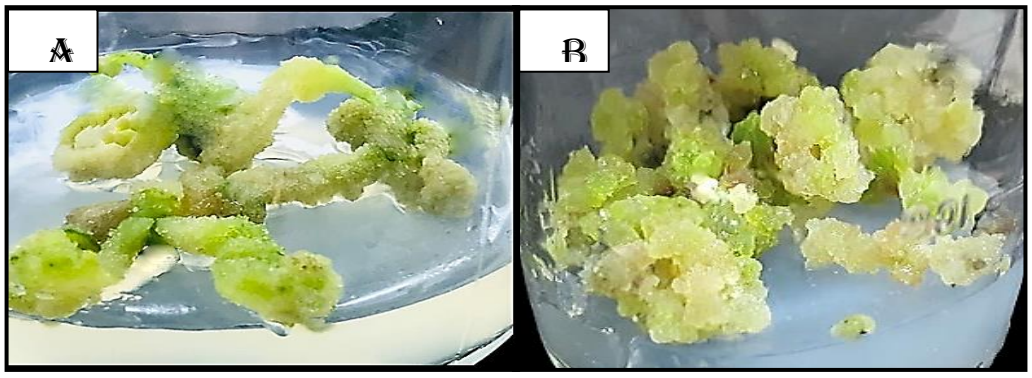


Figure (1): Production the callus cultures of chamomile (*Matricaria chamomilla* L.) grown on solidified MS medium supplemented with the addition of 1.0 mg L^{-1} of NAA, BA and 2,4-D

A: Callus initiation from hypocotyl stems segments of chamomile seedlings after 10 days

B: Continued growth in the size of callus masses in (A) and production its culture after 30 days.

The addition of auxins to the nutrient media, alone or mixed with Benzyl.adinen BA, had a prominent role in generating callus from cuttings of hypocotyl stems [26] and this is similar to the results of the study conducted by the researchers [27]. The behaviour of plant cuttings to generate callus depends on the compatibility between their internal content of plant hormones and growth regulators added to the medium [28].

Spontaneous formation of vegetative branches from the callus of chamomile (*Matricaria chamomilla* L.):

One of the distinctive results in this study is that 11 days after the first subcultured of callus on the same solid MS medium (Murashige and skoog, 1962) supplemented with of 1.0 mg L^{-1} from each of Naphthaleneacetic acid NAA, Benzyl.adinen BA and 2,4-Dichlorophenoxyacetic acid 2,4-D, appeared some vegetative growths which later developed into vegetative shoots with 95% and 12 branch\pieces of callus after 30 days (Figure, 2) and it carries real leaves.

Figure (2): Spontaneous differentiation of vegetative shoots from callus grown on solid MS medium contain of 1.0 mg L^{-1} form each of NAA, BA and 2,4-D after 30 days.

The spontaneous formation of vegetative shoots on the same callus growth medium may be attributed to the genotype of the plant variety used and

evidence of the totipotency of its cells in forming vegetative shoots, in addition to the levels of growth regulators within the callus tissue[29].

Efficiency the direct injection of seedling hypocotyl stem of chamomile by Ri vector plasmids isolated from *A.rhizogenes ATCC 15834* in the development of hairy roots and vegetative shoots:

The results of inoculating the seedlinge hypocotyl stems of chamomile plants with 1214.32 and 1159.24 ng μl^{-1} of Ri plasmids isolated from *A.rhizogenes ATCC 15834* and grown on solid MSO medium, showed variation in response to hairy roots and vegetative shoots produced with different percentages of them (Table, 1)

Table (1): Hairy roots and vegetative shoots production from seedling hypocotyl stems of chamomile inoculation with Ri vector plasmids isolated from *A.rhizogenes ATCC 15834* at different concentrations after 20 days.

Concentrations of Ri plasmids isolated from the vector plasmids of *A.rhizogenes* ATCC 15834 (ng μl^{-1})	Percentage of hairy root formation (%)	Number of inoculated / responding pieces	Percentage of vegetative branch formation (%)	Number of vegetative branches/ Piece	Period of vegetative shoots production (day)
1214.32	80	90\85	94	22	20
1159.24	50	90\50	55.5	15	20
(D.W)	0	90\0	0	0	0

The data showed superiority in the concentration of 1214.32 ng μl^{-1} of vector plasmid recorded in the highest rate of hairy roots production with 80% after 7 days and vegetative shoots at 94% after 20 days (Figure, A-4). Then the concentration of 1159.24 ng μl^{-1} enhanced stimulated 50% hairy roots and vegetative shoots with 55.5% after 20 days (Figure, B-4).

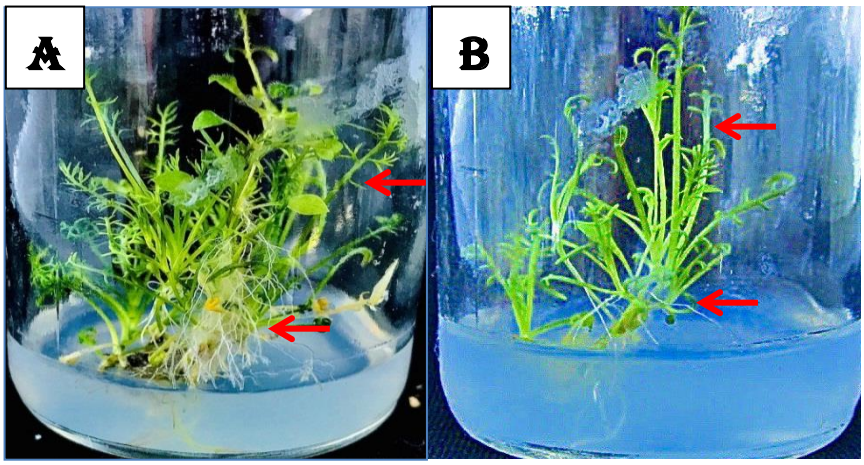


Figure (3): Hairy roots and vegetative shoots production from seedling hypocotyl stems of chamomile inoculation with Ri vector plasmids isolated from *A.rhizogenes ATCC 15834* at different concentrations

A- Hairy roots and vegetative shoots production with vector plasmids at 1214.32 ng μl^{-1} (arrow)

B- Hairy roots and vegetative shoots production with vector plasmids at 1159.24 ng μl^{-1} (arrow)

The success of the hairy roots and vegetative shoots produced belong to the success of the genetic transformation process of the chamomile plant, which means transfer of T-DNA genes from the bacterial plasmid vector into the genome of the plant cells [30], and the ability of this tissues for injection and the virulence of bavteria [31]. The hormonal imbalance caused by T-DNA genes is the reason for the formation of vegetative branches from the sites of pollination. It was found that the increase in the concentration of plasmid used to inoculation the leaves had a direct effect on increasing the efficiency and rate of the genetic transformation, which. As well as the difference in the response of the plant parts used [32].

Apigenin content in different tissues of chamomile plants using High-Performance Liquid Chromatography (HPLC) technique:

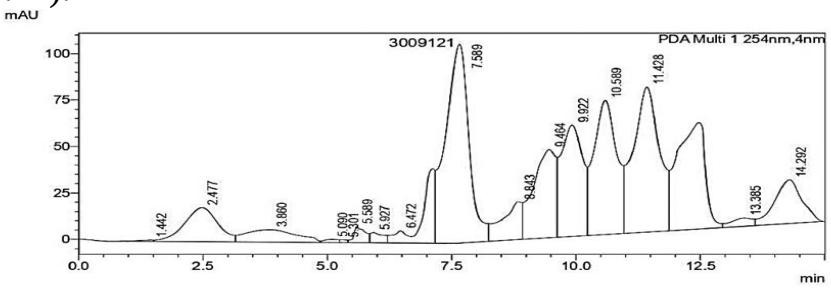
The curves for diagnosing the apigenin compound in various chamomile tissues and estimating its concentrations using an HPLC device showed different in its concentration (Table, 2).

Table (2): Retention time, area of the curve, and apigenin concentration in the different of vegetative shoots extracts of chamomile

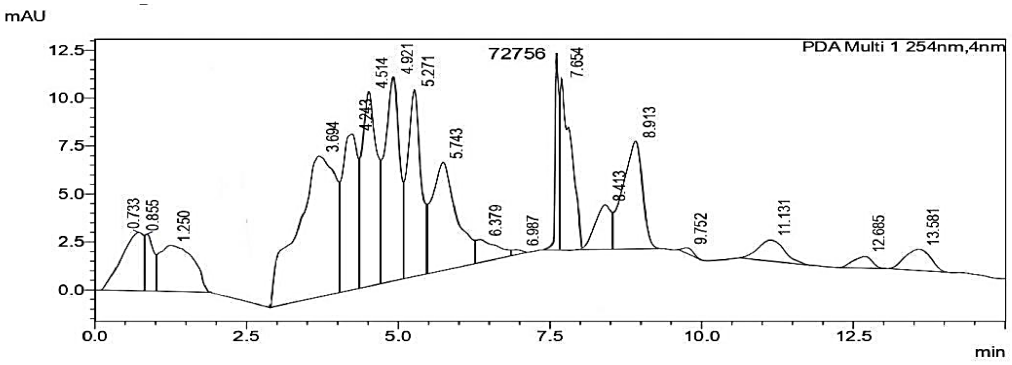
Vegetative shoots of the chamomile plant separated from	Retention time (min)	Curved area	Apigenin concentration (µg ml^{-1})
Apigenin standard	7.589	3009121	0.5
Wild plant	7.609	415482	0.0690
seedlings at 30 days old	7.654	72756	0.0121
differentiation form callus	7.677	551398	0.0916
Transgenic seedlings with Ri plasmids of *A. rhizogenes* ATCC 15834 with concentration 1214.32 ng μl^{-1}	**7.562**	**2678573**	**0.4451**

With the superiority of the apigenin content in tissues genetically transformed with 1214.32 ng μl^{-1} of Ri plasmids vector isolated from bacteria *Agrobacterum rhizogenes ATCC 15834*, the concentration reached to 0.4451 µg ml^{-1} with curve was 2678573 (Figure, 3. E). Then its content in the other vegetative shoots tissues differentiation form callus, wild plant and seedlings at 0.0916, 0.0690 and 0.0121 µg ml^{-1} and curve with 551398, 415482 and 72756 respectively (Figure, 3. D-B-C).

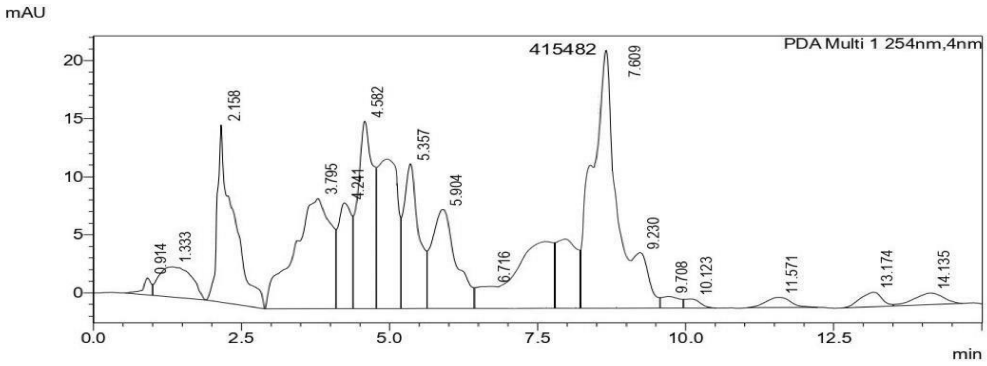
While the curve of standard apigenin compound a resulted from injecting a sample of the into (High-Performance Liquid Chromatography) HPLC showed the largest area ratio of the apigenin curve which reached 3009121 (Figure, 3. A).



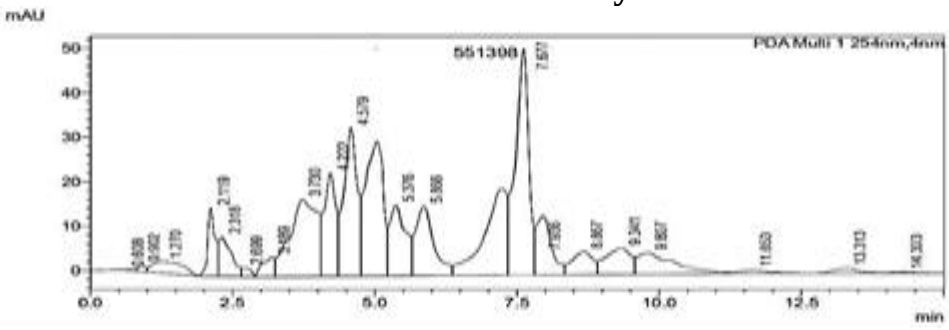
A: Apigenin standard curve



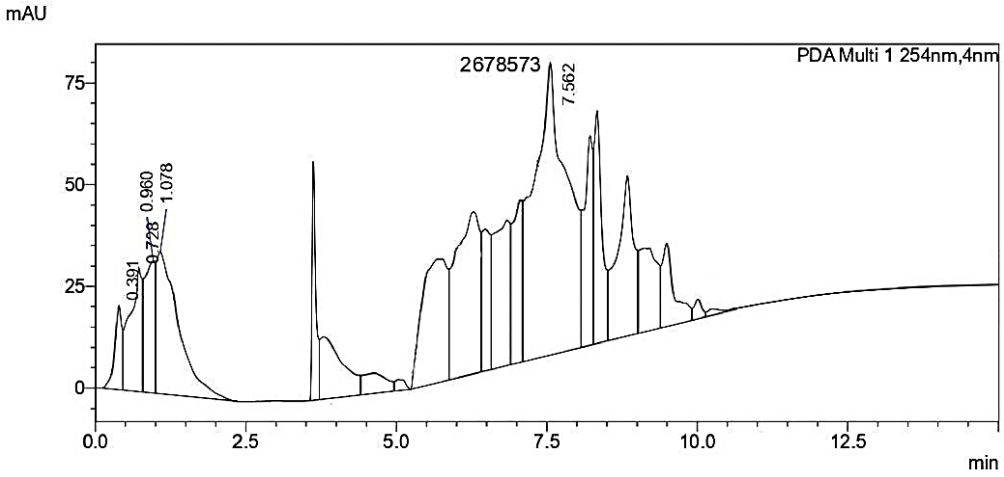
B: Apigenin curve in the vegetative shoots of wild chamomile



C: Apigenin curve in the vegetative shoots of chamomile seedlings branches after 25 days old.



D: Curve of apigenin isolated from the vegetative shoots differentiation form callus grown on MS medium contain 1.0 mg L^{-1} from each of NAA, BA and 2,4-D after 25 day old



E: Apigenin curve in vegetative shoots of genetically transformed chamomile plants with Ri plasmids vector **at 1214.32 ng µl^{-1} after 28 day old**

Figure (3): Apigenin curve in different types of vegetative shoots of chamomile plants by HPLC technique

The high content of apigenin in transgenig vegetative shoots by Ri vector plasmids isolated from *A.rhizogenes ATCC 15834* at the two-fold compared with non-transgenic tissues may belong to the successful transferred of the *rol* genes present within the T-DNA segment from the plasmid to the plant cells genome and integrated in it. Their integration into their genetic material led to increased expression of the genes responsible for the biosynthesis process of apgenin, or to genetic stability and increased cell divisions caused by the genetic expression of apgenin genes [33]. As well as, the control of stable genes in the cells of the genetically transformed tissues, in addition to the high rates of differentiation of vegetative branches, and the superiority of the tissues genetically transformed with Ri vector plasmids in producing larger amounts of secondary metabolite compounds [34].

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تقدير محتوى الأبيجينين في أنسجة الأفرع الخضرية المختلفة لنباتات البابونج
(*Matricaria chamomilla* L.)

مستخلص البحث:

نجحت النتائج في استحداث الكالس من سيقان تحت الفلقية من شتلات البابونج (*Matricaria chamomilla* L.) الصلب (*Murashige* and *skoog*,1962) MS على وسط المضاف إليه إضافة 1.0 مجم L^{-1} من كل من حمض النفثالين أسيتيك (NAA، 6-بنزيل أمينوبورين (BA) و 2،4- حمض ثنائي كلورو فينوكسي أسيتيك (D-2،4) بنسبة تصل إلى 100% بعد 30 يوماً. واستمر نمو الكالس حتى أنتجت مزارع نموذجية وتمايزها للأفرع الخضرية. كما توصلت هذه الدراسة إلى تحقيق التحول الوراثي لنبات البابونج مع ناقل بلازميد Ri المعزول من بكتريا *Agrobacterium rhizogenes ATCC 15834* باستخدام تقنية الحقن المباشر، مع تفوق بتركيز 1214.32 نانوجرام ul^{-1} في نسبة الجذور الشعرية المنتجة بنسبة 80% والأفرع الخضرية.

حددت النتائج محتوى الأبيجينين في الأنسجة المختلفة للبراعم الخضرية لنبات البابونج باستخدام تقنية تحليل كروماتوغرافيا السائل العالي الأداء (HPLC)، حيث بلغ التركيز العالي 0.4451 ميكروغرام مل-^{1} في الأنسجة المحولة وراثيا مع ناقل بلازميدات Ri عند 1214.32 نانوغرام أول-^{1} وتفوقت على العينات الأخرى وكانت أقل نسبة من الأبيجينين في الأفرع الخضرية التي تميزت عن الكالس بنسبة 0.0121 ميكروجرام مل-1.

الكلمة المفتاحية: البابونج الالماني، التحول الوراثي، الأبيجينين، HPLC.