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EFFECT OF GENETIC TRANSFORMATION VIA *AGROBACTERIUM RHIZOGENES* ON THE ANATOMICAL CHARACTERISTICS OF *ERUCA SATIVA* MILL. PLANTS

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ABSTRACT

This study is conducted to find out the effect of genetic transformation by *Agrobacterium rhizogenes* ATCC13332 on the anatomical features of *Eruca sativa*. Stem explants and leaves were inoculated by direct injection of *A. rhizogenes*. The results indicated the high response of *E. sativa*, as the percentage of hairy roots formation was 95.3% for leaves. Callus was successfully initiated from hairy roots on both Murashige-Skoog (MS) and Woody plants (WP) media containing (0.4 and 0.5 mg. L^{-1} thidiazuron(TDZ). Shoot regeneration occurred spontaneously. Also, the callus originated from hairy roots showed ability of shooting. Genetic transformation was detected by polymerase chain reaction (PCR). Alterations in anatomical structure of stems and leaves in transformed plants were observed. Transgenic plants' epidermal cell thickness was 14.3 µm rather than 12 µm. There are nine vascular bundles in regenerated plants that are open collateral vascular bundles. The palisade tissue thickness of transformed leaves was 96 µm, while 66 µm in non-transformed. It is clear that the genetic transformation led to anatomical changes of this medicinal plant.

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INTRODUCTION

The cloning, delivery, integration, and expression of target genes, as well as the regeneration, screening, and identification of transgene-positive plants, are all examples of genetic transformation, a supporting technique for genetic engineering (Feng *et al*., 2023). *Agrobacterium rhizogenes*, as it is now known taxonomically *Rhizobium rhizogenes* is a gram-negative soil bacterium lives close to plant roots and is eventually responsible for the so-called "hairy root syndrome" in the infected plant host. A non-geotropic branching root outgrowth at the infection site characterizes this condition**.** (Gutierrez-Valdes *et al*., 2020). The root-inducing (Ri) plasmid containing transfer DNA(T-DNA) encoding *root locus* (*rol*) gene loci (*rolA*, *rolB*, and *rolC*), is in charge of introducing genetic material into host plant cells in a steady manner (Ron *et al*., 2014). The *rol* genes play a crucial part in the development of this hairy root (Nemoto *et al*., 2009). Different species of these bacteria are employed in biotechnology to insert genes into plants and improve traits. The hairy root *rol* genes are present with transgenes after transformation and modify the morphology of plants (Lankitus *et al*., 2023).

The annual herbaceous plant species of arugula *E. sativa* commonly known as rocket salad belongs to the Brassicaceae family and is distinguished by its strongly

flavorful and pungent leaves (Hall *et al*., 2015). It is raised as an oilseed, vegetable, and medicinal crop (Sharma, 2012). This plant's seeds, oil, and leaves have been utilized for a variety of therapeutic reasons since ancient times. It improves the lipid profile (Abdul-Majeed and Taha, 2019), liver function, boosts bile production, aids in digestion, and treats urinary and gastrointestinal disorders (Al-Shammarie and Batkowska, 2021).

E. sativa plant contains antioxidant glucosinolates, flavonoids, and vitamins A and C, which have been demonstrated to enhance growth metrics and blood characteristics. Effective oxidation combats stress, free radical production, and harmful bacteria, improving fish health and encouraging development and food use (Al-Rawe *et al*. 2023). Rocket consumption has significantly increased recently, making it crucial for breeders. A platform for overcoming the challenges in advancing this species is provided by plant tissue culture (Banjac *et al*. 2023).

Anatomical features have an important role in classifying plant species. Different parts of plant were used as a taxonomic tool includes fruits and seeds (Saeed, 2022). Few studies have investigated genetic transformation of rocket salad by Agrobacterium (Kastell *et al*., 2018; Park *et al*., 2021). However, the anatomical characteristics of *E. sativa* have not been covered in previous studies. Hence, this work is a trial to estimate the impact of transformation by *A. rhizogenes* ATCC13332 at the anatomical level in this important plant.

MATERIALS AND METHODS

Plant material

All the experiments of the research were carried out in laboratories of Research Unit, Biology Department/ College of Education for Pure Sciences / Mosul University. Rocket salad seeds were obtained from local market, then surface sterilized according to method of Park *et al*. (2021). The seeds were immersed for 30 seconds in ethanol (70%), after that, immersing in 2% NaOCl for ten minutes. Then *E. sativa* seeds were dipped 5 times in axenic distilled water. The seeds were planted in 100 ml glass vials containing 20 ml agar- solidified MS (Murashige and Skoog, 1969) medium free from growth regulators. Samples were kept in growth room under conditions at 25±2ºC, 16 h photoperiod.

Preparation of bacterial inoculum

A.rhizogenes ATCC13332 carrying the Ri plasmid was obtained from Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures. A single colony of the bacteria was collected and transferred to 25 mL Nuttrient Broth. Liquid culture was put in the shaker incubator (28◦C, 150 rpm). By measuring optical density (OD) at 600 nm, bacterial division was verified and two concentrations of inoculum (OD_{600} 0.7 and 1.6) were used in the inoculation process.

Direct inoculation of explants

Stems and leaves of 15 days old axenic seedlings were inoculated directly using an insulin syringe, the end of which was immersed in the bacterial suspension. The leaves were inoculated in the midrib. All the specimens were transferred to both hormone-free MSO and WPO (Woody Plant) medium (Lloyd and Mccown, 1980) and incubated at 25±2ºC under complete darkness conditions.

Initiation of transformed hairy root cultures

The induced hairy roots were excised from the stems and leaves, and then transferred to agar solidified MSO and WPO medium containing 200 mg/L cefotaxime. This process was repeated every 20 days. The hairy root cultures were evaluated after two months of culture.

Callus induction from hairy roots

For callus initiation, pieces of hairy root approx.1-2 cm grown on both MSO and WPO medium were cultured on 25 ml of the following callus induction media (CIM) in glass jars:

CIM1: MS containing 0.4 mg L^{-1} thidiazuron (TDZ)
CIM2: MS + 0.5 mg L^{-1} TDZ
CIM3: WP + 0.4 mg L^{-1} TDZ
CIM4: WP + 0.5 mg L^{-1} TDZ

The samples were kept under conditions of 25±2ºC, 16 h photoperiod in growth room. The data were collected after two months.

Maintenance of hairy root and callus

Hairy roots were sub-cultured every 20 days by transferring clusters of them to fresh MSO, while the callus was maintained via sub-culturing on CIM3 medium every 20 days at 25±2ºC, 16 h photoperiod.

Shoot regeneration and acclimatization

During the process of maintenance hairy roots, shoots were regenerated spontaneously at the same medium of subculture. In addition, calli produced from hairy roots had the ability of shooting at the same medium of callus induction. All these regenerated shoots were carefully excised and transferred to MSO medium for rooting. Afterward, regenerated plantlets were transferred to pots (Ø 9 cm) containing a mixed of sterilized soil and peat moos and covered with perforated plastic bags. After ten days, the plastic covers were removed and the regenerated plants were transferred to greenhouse conditions.

Molecular detection of *rol-C*

DNA was extracted from 100 mg of the following plant tissues: regenerated plants, hairy roots and seed -born plants, according to Healey *et al*. (2014) using hexadecyltrimethylammonium bromide (CTAB):

DNA purity was determined using the NanoDropND-1000 spectrophotometer. Then, amplified by PCR, the *rol* gene primers of Hom-utai (2009) were selected:

F=5′CATTAGCCGATTGCAAACTTG3′
R=5′ATGGCTGAAGACCTG3′

Premix tubes were used containing 5.0 µl of the mixture ($MgCl_2$ buffer+ KCl + Tris-HCl + dNTPs+ Taq DNA polymerase enzyme)1.0 microliters of the forward primer with a concentration of 10 pmol/µl, the reverse primer, 4.0 µl of DNA template with a concentration of 50 ng/µl were added, in addition to 9.0 µl of sterile deionized water, and at the end of the reaction time, 3 µl were taken from each of them, and they were loaded into a double 2% agarose gel. A digital camera was used to photograph the separated bands.

Anatomical analysis

Anatomical characterizations of leaves and stems (1-2 cm long) of regenerated plants and seed-born plants were performed. Procedure of Gomes *et al*. (2017) was followed with some modification. Samples were collected from three-month-old plants; they were immersed in a killing and fixation solution of FAA (5ml formaldehyde, 5ml glacial acetic acid and 90ml of ethanol 70%) for 24 hours. Afterward samples were washed twice with 70% ethyl alcohol, and then dehydrated through passing in a series of ethyl alcohol. The samples were placed in paraffin wax after that, cutting using microtome. Finally, stained with Safranin dye and fast green, afterwards, they were cleared in xylene and mounted in Canada balsam. Then, examine with a light microscope. The data were recorded for different anatomical characteristics and the standard deviation was calculated.

RESULTS AND DISCUSSION

Induction of hairy root by direct injection

The results indicated the success of direct injection of stems and leaves of *E. sativa* with *A. rhizogenes* ATCC13332 Table (1). As the hairy roots appeared after 16 days on the inoculated and un-inoculated sites of stems Figure (1, a).

Table (1): Induction of hairy roots on stem explants and leaves of *Eruca sativa* seedlings, inoculated with *A.rhizogenes* ATCC 13332.

Type of explant	Inoculation density O-D	Numbers of responsive/ inoculated	Induction rate %	Induction duration (day)
Stem explants	0.7	32/50	64.0	17
	1.6	37/50	74.0	16
	* control	0/25	0.0	0.0
Leaves	0.7	57/65	87.6	8
	1.6	62/65	95.3	8
	* control	0/30	0.0	0.0

*Explants were inoculated with distilled water.

Moreover, we attempted to increase the transformation effectiveness via optimizing the (OD_{600} 1.6) which was very effective since the percentage of hairy roots reached 74 and 95.3 % for stem and leaves respectively. In addition, the hairy roots appeared after 8 days of direct injection of leaves Figure (1, b). The hairy roots are bright white, thin and they were developed after 40 days to give clusters of high branches Figure (1, c & d).

Callus initiation and shoot regeneration

Results of culturing the hairy roots in callus induction media referred that both MS and WP media supplemented with thidiazuron were suitable for callus initiation Table (2). Furthermore, CIM3 (WP containing 0.4 mg. L^{-1} TDZ led to the best callus formation percentage (100%) after 20 days of culture. The process of callus formation began with the enlargement and thickening of the hairy roots, in addition to turning their color to green Figure (2, a, b).

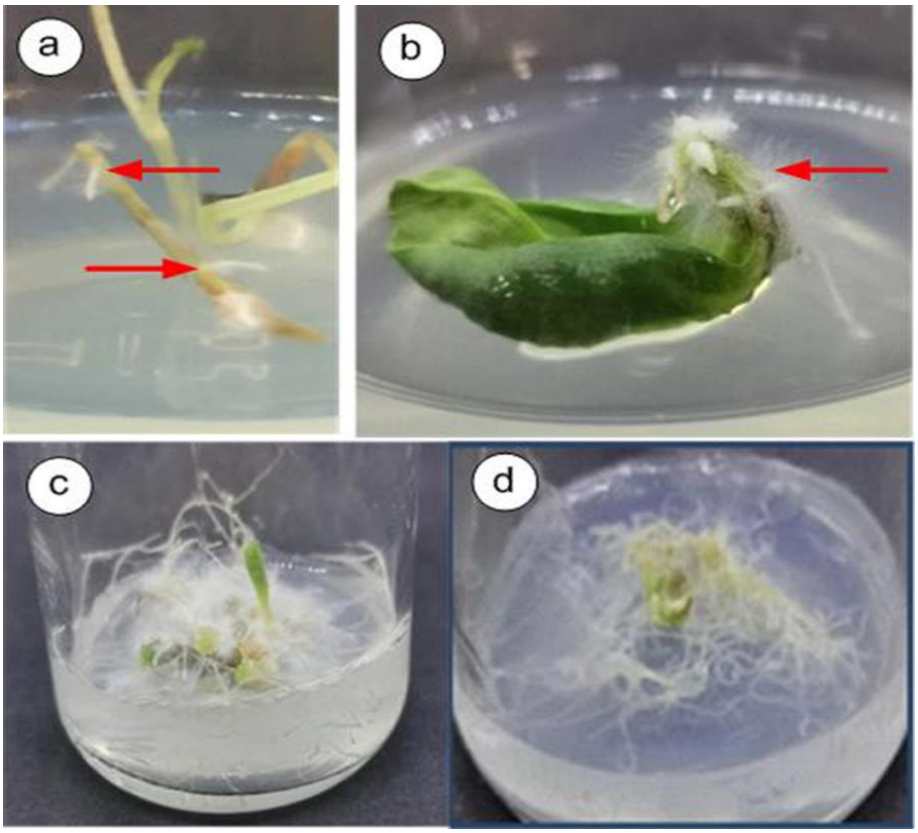

Figure (1): Initiation of hairy root culture of *E. sativa*. (a) Stem explant after 16 days of injection by *A. rhizogenes* ATCC13332. (b) The leaf after 8 days of inoculation. (c,d) Hairy roots cultures of both stems and leaves respectively after 40 days on MSO medium.

Table (2): Callus initiation and shoot regeneration from hairy roots of *E. sativa* on MS and WP medium

Medium	Number of hairy roots forming callus/ No. of cultured hairy roots	Induction of callus %	No. of regenerated shoots
CIM1	30/33	90.9	17
CIM2	36/38	94.7	19
CIM3	45/45	100	24
CIM4	38/40	95	23
(control) MSO	17/30	56.6	15
(control) WPO	0/30	0.0	0

One of the important results in this study is that segments of hairy roots growing on MSO medium formed masses of greenish white callus after four months Figure (2, c). The callus was successfully maintained on CIM3 medium every 20 days Figure (2, d).

Interestingly, shoot regeneration occurred spontaneously from hairy root during subculture and maintenance on free MSO medium. The regeneration of shoots started by formation of small leaves-like structures after 3 months, which developed to true leaves Figure (3, a).

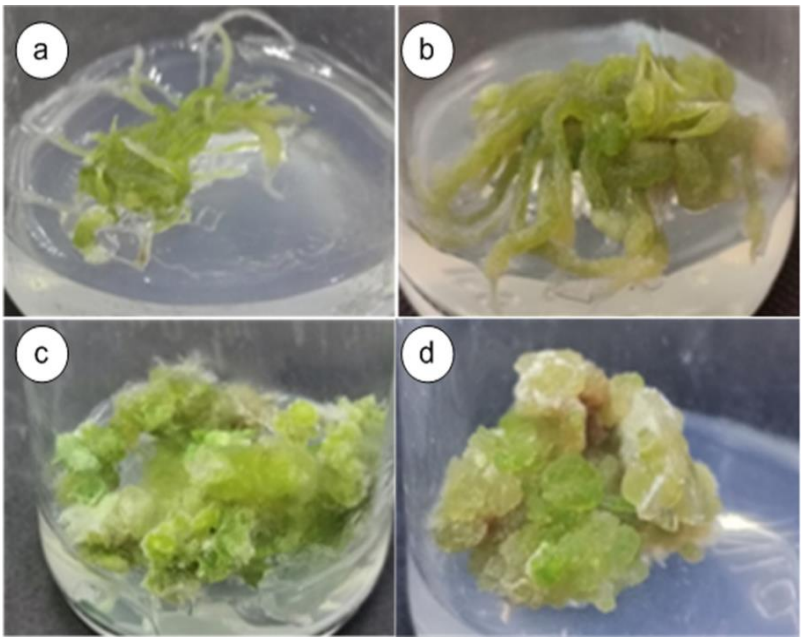

Figure (2): Callus induction from hairy roots of *E. sativa*. (a) Beginning of callus formation in CIM3 medium. (b) A mass of hairy roots in (a) after 30 days. (c) Callus originated from hairy roots after 3 months of subculture on MSO (d) Callus of hairy roots on maintenance CIM3 medium.

Moreover, the callus originated from hairy root showed a high ability to form shoots at the same medium of callus initiation after 30-40 days of sub-culture Figure (3, b). Regenerated shoots rooted easily in MS medium free from growth regulators Figure (3, c), and transformed plant were acclimatized successfully to soil condition Figure (3, d).

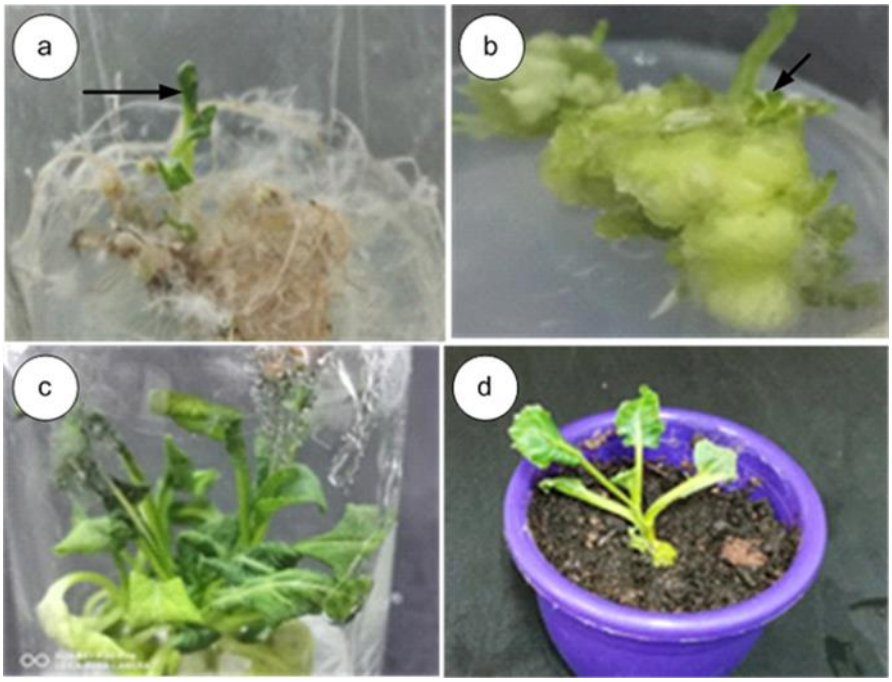

Figure (3): Plant regeneration from hairy root cultures of *E. sativa*. (a) Spontaneously shoot regeneration from hairy roots after 3 months on MSO medium (arrow) (b) Shoot regeneration from callus originated from hairy roots on CIM3 medium (arrow) (c) Rooting of regenerated shoots (d) Acclimatization of regenerated plants.

Detection of *rol*C

The PCR analyses of the transformed tissues produced the amplification of a 545-bp fragment (*rol*C), which is comparable to the positive control's (plasmid DNA from ATCC 13332 amplified product). The DNA recovered from seed-born plants, however, did not contain such an amplification product Figure (4).

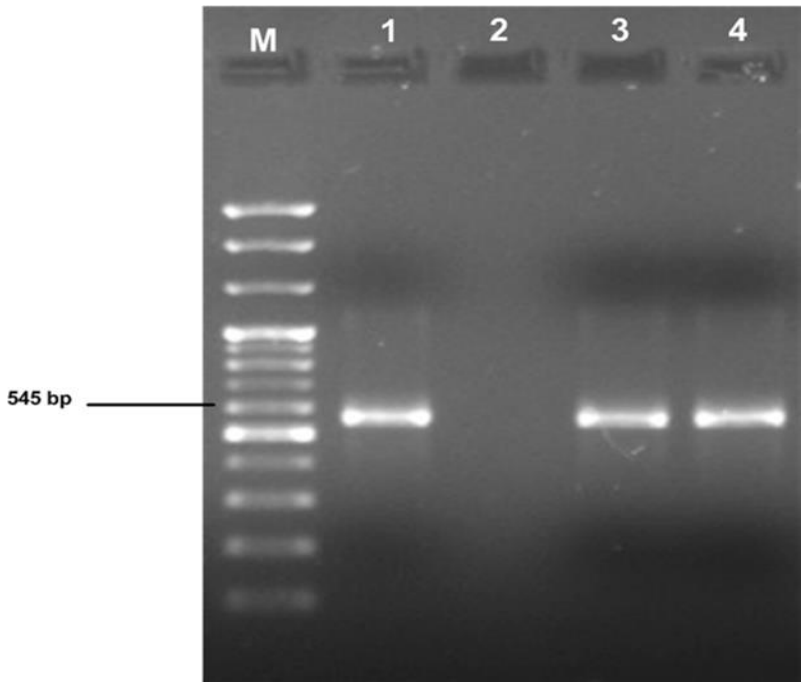

Figure (4): PCR analysis of transformed tissues of *E. sativa*. M: (100-bp DNA ladder), Lanes: 1 positive control (plasmid DNA from ATCC 13332 strain), 2 negative control (genomic DNA from seed-born plant), 3 genomic DNA of transformed plants, 4 genomic DNA of hairy roots, arrow referred to molecular weight in base pair (bp).

Histological analysis

The observation of anatomical features of *E. sativa* stems and leaves showed alterations in internal structure of cross sections of regenerated plants, as compared to those of seed –born plants Table (3).

Table (3): Anatomical characteristics of transformed and seed-born plants of *E. sativa.*

Characteristics parameters ± SD	Seed –born plant	Transformed Plant
Thickness of Cuticle in stem (µm)	6.3 ± 0.122	3.5 ± 0.254
Epedermis thickness of stem(µm)	12 ± 0.707	14.3± 1.346
Cortex thickness of stem(µm)	30 ± 0.707	35 ± 1.274
No. of vascular in stem bundles	7 ± 0.471	9 ± 0.707
No. of xylem arms/ bundle in stem	3-7	5-8
Thickness of palisade tissue	66± 2.88	96±1.414
Spongy tissue thickness	60±1.414	87±1.22

The thickness of stem epidermal cells for transgenic plants was 14.3 µm compared to 12 µm for seed-born plants. In general, the epidermis consists from single row of small irregular cells. The cortex thickness was 35 µm in transformed plant compared to30 µm for seed plant. However, it consists from parenchyma cells serve as storage tissue. Moreover, the number of vascular bundles which are open collateral vascular bundles for regenerated plants was 9 Figure (5, a) compared to 7 for seed-born plants Figure (5, b).

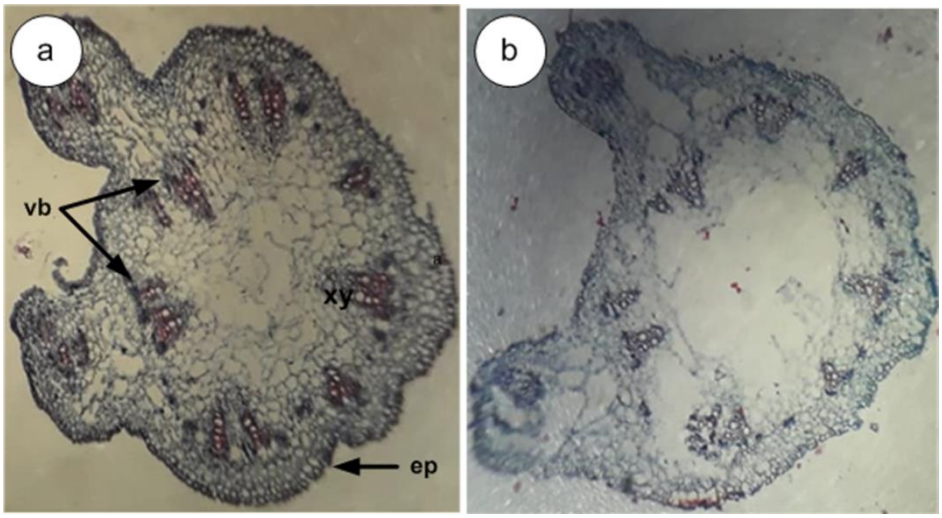

Figure (5): Cross sections of *E. sativa* stem (a) Transformed plant, vb= vascular bundles, ep = epidermis, xy= xylem. (b) Seed-born plant.

On the other hand, anatomical differences were observed between cross-sections of leaves of transgenic plants Figure (6, a) and those of seed plants Figure (6, b). The leaf of *E. Sativa* is unifacial has a palisade parenchyma tissue consist from elongated cells located under the upper epidermis. The leaf mesophyll's lower epidermis is composed of spongy cells. The thickness of palisade tissue in transformed plant was 96 µm compared to 66 µm in non-transformed Table (3). The vascular bundles are of closed collateral type present in the central part, consisting of phloem and xylem.

Genetic transformation by *A. rhizogenes* in plants is considered a good procedure which results in appearance of hairy roots as a result of changing endogenous auxin/cytokinin ratio in the plant cell (Palazón *et al*., 1998). Furthermore, it is widely known that, hairy roots can be grown on growth regulators-free medium (Gunjan *et al*., 2013). In our study, *E. sativa* responded successfully to direct injection by *A. rhizogenes* ATCC13332. The hairy root induction percent in leaves was 87.6% compared to 64.0% for stem explant. This is may be due to the differences in the internal content of hormones and the numbers of cells that respond to the direct injection. In addition, the inoculation of the leaves takes place in their midribs, which are known to have a large number of parenchyma cells. It was reported that, there are various plant parameters, including the type of explants (Khlifa *et al*., 2020), the cultivar, and the culture conditions, could have an impact on the success of the transformation (Michalec-Warzecha *et al*., 2016). Moreover, the inoculum density of *Agrobacterium* considered another factor which affecting transformation of plant. Therefore, if *A. rhizogenes* concentration is low, the capacity to infect is weak and

the amount of hairy roots produced is significantly reduced (Satish *et al*., 2017).

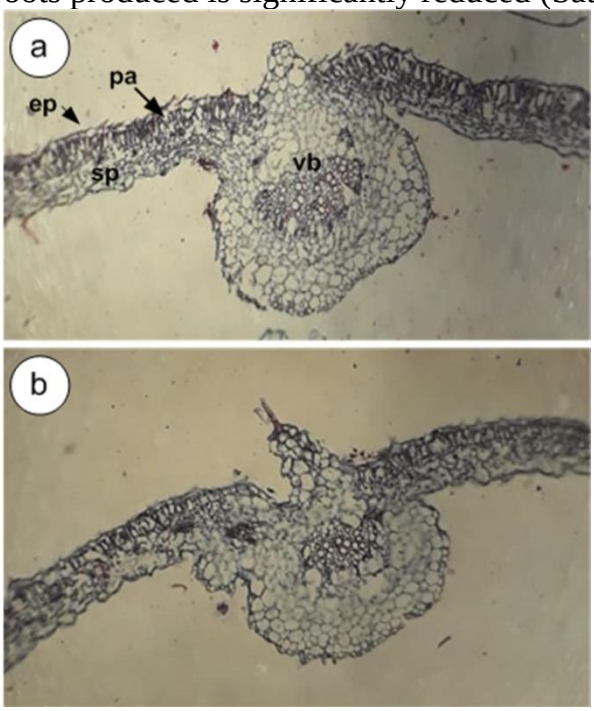

Figure (6): Cross sections of *E. sativa* leaf (a) leaf of transformed plant, vb= vascular bundles, ep = epidermis, pa= palisade tissue, sp= spongy cells (b) leaf of seed-born plant.

This explains the increase in the percentage of hairy root formation Table (1), when we used (OD_{600} 1.6) of the inoculum. According to studies, hairy roots are regarded as the initial indicators of genetic change (Ionkova and Fuss, 2009; Komarovská *et al*., 2009). It was very clear, that the hairy roots had the ability to grow on MS and WP media free from growth regulators. This is a main characteristic of hairy roots, which was approved by other researchers whom they confirmed that, hairy roots can grow in medium free from phytohormone (Chaudhuri *et al*., 2006; Gunjan *et al*., 2013).

Interestingly, callus was initiated successfully, when the hairy roots were cultured on both MS and WP media containing TDZ. The active phenyl urea molecule TDZ has an important function as a regulator of plant growth, its ability to replicate auxins and cytokinin while being chemically distinct from these two is its most intriguing features (Ali *et al*., 2022). Sharafi *et al*. (2014) reported that hairy roots of *Dracocephalum kotschyi a* medicinal plant induced by *A. rhizogenes* ATCC 15834 had the ability to initiate callus in MS medium containing 0.1 0.25, 0.5 $mg.l^{-1}$ of benzyl adenine (BA) and0.1 $mg.l^{-1}$ NAA after 2 weeks. However, during subculture of the hairy roots on MSO medium, callus was produced. This result may be due to the transformation since the infection by *Agrobacterium* alters the endogenous auxin/cytokinin ratio.

One of the distinctive results in this study, was the spontaneous regeneration of (15) shoots from hairy root culture through maintenance the hairy roots on free MSO medium. Observations of spontaneous shoot organogenesis in hairy root cultures of some medicinal plants were recorded including, *Lopezia racemosa (*Vargas Morales *et al*., 2022), *Plumbago indica* (Gangopadhyay *et al*., 2010) and *Tylophora indica*) Chaudhuri *et al*., 2006). In addition, shoot regeneration were

observed during callus culture of hairy roots. According to reports, shoot regeneration happened as a result of the overexpression of the *rol*C genes found on the *A. rhizogenes* Ri plasmid's cytokinin-mimetic action (Casanova *et al*., 2003).
The detection of *rol*C genes in the transformed tissues of *E. sativa* demonstrated the efficient integration of genes from *Agrobacterium* into plant genome. The advantage of using PCR to characterize transgenic plants is that the newly inserted genes can be found earlier with less DNA and plant material (Vergauwe *et al*., 1996). Moreover, this technique is a quick and simple method that adopted for different applications (Abdulrazaq,2022). The T-DNA is inserted randomly into the genome of the recipient plant cell during genetic transformation (Rajagopalan and Perl-Treves 2005).

Concerning the differences in anatomical characteristics which are recorded in this work, and according to our information and after research, we did not find previous studies on the effects of genetic transformation on the anatomy of *E. Sativa*. However, Lankitus *et al*. (2023) indicated that the physiological characteristics and morphology of rubber dandelion have been significantly affected by the hairy root *rol* genes. The increase in the thickness of stem and leaf tissues of transformed plant especially the palisade tissue Table (3) may reflect positively on many aspects of the plant, especially the process of photosynthesis. Thickness mesophyll tissue will lead to increase the mesophyll surface area and ultimately increasing the rate of photosynthesis. Terashima *et al*. (2011) reported that, in high-light settings, thick leaves with a large mesophyll surface area are useful for achieving high photosynthetic rates.

CONCLUSIONS

In this study, the successful application of *A. rhizogenes* ATCC13332 was reported, with the important medicinal plant *E. sativa* Mill as the hairy root production rate reaching 95.3%. The fast growth of hairy roots on MSO medium and the easy regeneration with high percentage, which are achieved in this research, will aid the utilization to use this medicinal plant in different aspects. Moreover, the changes in some anatomical features in the transformed plants probably, reflect on different physiological processes. Thus, improving the plant and raising the efficiency of its production of medicinal compounds.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

تأثير التحول الوراثي ببكتريا *Agrobacterium rhizogenes* على الخصائص التشريحية لنبات الجرجير *Eruca sativa* Mill.

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الخلاصة

اجريت الدراسة الحالية لايجاد تأثير التحول الوراثي ببكتريا *Agrobacterium rhizogenes* ATCC13332 على الخصائص التشريحية لنبات الجرجير. لقحت قطع السيقان والاوراق باستخدام الحقن المباشر ببكتريا *A. rhizogenes* واظهرت النتائج الاستجابة العالية للتحول الوراثي حيث بلغت نسبة تكوين الجذور الشعرية 95.3% للاوراق. تم استحداث الكالس من الجذور الشعرية في كل من وسط موراشيج وسكوك (MS) ووسط (WP) المجهزين بـ 0.4 و0.5 ملغم.لتر$^{-1}$ thidiazuron (TDZ). تكونت الافرع الخضرية بشكل تلقائي من الجذور الشعرية، فضلاً عن تكونها من الكالس الناتج من الجذور الشعرية. تم تأكيد التحول الوراثي باستخدام تفاعل البلمرة المتسلسل (PCR). وأشارت النتائج الى حصول تغيرات في التركيب التشريحي لسيقان وأوراق النباتات المحولة وراثياً، اذ بلغ سمك خلايا البشرة للسيقان 14.3 ميكرومتر مقارنة بـ 12 ميكرومتر للنباتات البذرية (المقارنة). لوحظ ايضاً وجود 9 حزم وعائية من نوع الحزم الجانبية المفوحة في سيقان النباتات المحولة وراثياً في حين بلغ عددها 7 في النباتات البذرية.وبلغ سمك النسيج العمادي لاوراق النباتات المحولة وراثياً 96 ميكرومتر مقارنة بـ 66 في نباتات المقارنة. اوضحت هذه الدراسة التي تعد الاولى التي تناولت تاثير التحول الوراثي على التركيب التشريحي لنبات الجرجير ان عملية التحول الوراثي تؤدي الى تغيرات في الصفات والخصائص التشريحية للنبات والتي يمكن تطبيقها مع نباتات اقتصادية مهمة تساهم في تحمل هذه النباتات الى الظروف البيئية غير الملائمة.

الكلمات المفتاحية: كالس، جذور شعرية، أوراق، تحول.

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