Zinc Oxide Nanoparticles Induce Genetic Transformation of *Silybum marianum L.* Mediated by *Agrobacterium rhizogenes* ATCC13332

Nanopartículas de óxido de zinc inducen la transformación genética de Silybum marianum L. mediada por *Agrobacterium rhizogenes* ATCC13332

Islam Yasir Abdullah Al-Hamdany[1], **Shifa M. Salih**[2*], **Muthanna Jasim Mohammed**[3].

Highlights

- Zinc oxide nanoparticles significantly enhanced Agrobacterium rhizogenes-mediated genetic transformation efficiency in *Silybum marianum*.
- Hairy root-derived callus induction reached 100% on MS2 and WP-based media supplemented with auxins and cytokinins.
- rolB gene integration was confirmed by PCR in transformed roots, shoots, and calli, validating stable T-DNA transfer.

Innovaciencia
ISSN: 2346-075X

E- ISSN: 2346-075X
Innovaciencia 2025; 13(1); e5211
http://dx.doi.org/10.15649/2346075X.5211

ORIGINAL RESEARCH

How to cite this article:

Al-Hamdany I.YA., Salih, S.M., Mohammed MJ. Zinc Oxide Nanoparticles Induce Genetic Transformation of *Silybum marianum L.* Mediated by *Agrobacterium rhizogenes* ATCC13332. Innovaciencia 2025; 13(1); e5211.
http://dx.doi.org/10.15649/2346075X.5211

Received: 30 May 2025
Accepted: 25 September 2025
Published: 27 October 2025

Keywords:

Agrobacterium rhizogenes; milk thistle; nanoparticles; rolB.

Palabras clave:

Agrobacterium rhizogenes; cardo mariano; nanopartículas; rolB.

ABSTRACT

Introduction. Nanoparticles have recently garnered substantial interest across various scientific disciplines due to their novel physicochemical properties. These particles have found broad applications in medicine, agriculture, environmental sciences, and pharmaceutical technologies. **Objectives.** To investigate the effect of zinc oxide nanoparticles (ZnO NPs) on the transformation efficiency of the medicinal plant milk thistle (*Silybum marianum L.*) mediated by *Agrobacterium rhizogenes*. **Materials and Methods.** Explants derived from 20-day-old seedlings were directly injected with *Agrobacterium rhizogenes* ATCC13332 using a 0.5 mL insulin syringe. Treated explants were cultured on solidified Murashige and Skoog (MS) medium devoid of growth regulators. **Results.** Hairy roots were induced in 55.86% of leaf explants, marking the first visible indicator of successful genetic transformation. Callus induction from hairy roots reached a 100% success rate on MS2 medium (MS + 1.0 $mg\cdot L^{-1}$ benzyladenine [BA] + 0.1 $mg\cdot L^{-1}$ indole-3-butyric acid [IBA]). Polymerase chain reaction (PCR) analysis confirmed the presence of the rolB gene in the genomic DNA of transformed tissues. Shoot regeneration occurred during hairy root maintenance. Furthermore, supplementing MS medium with varying concentrations (25–300 µg/mL) of ZnO NPs significantly enhanced hairy root induction, achieving a maximum rate of 100% in leaf explants cultured on MS medium containing 150 µg/mL ZnO NPs. **Conclusions.** Zinc oxide nanoparticles effectively enhanced the genetic transformation frequency in *S. marianum*, demonstrating their potential as a nanobiotechnological tool in plant genetic engineering.

RESUMEN

Introducción. Las nanopartículas han captado recientemente un notable interés en diversas disciplinas científicas debido a sus propiedades fisicoquímicas novedosas. Estas partículas presentan amplias aplicaciones en medicina, agricultura, ciencias ambientales y tecnología farmacéutica. **Objetivos.** Investigar el efecto de las nanopartículas de óxido de zinc (ZnO NPs) sobre la eficiencia de transformación genética de la planta medicinal cardo mariano (*Silybum marianum L.*), mediada por *Agrobacterium rhizogenes*. **Materiales y métodos.** Se utilizaron explantes de plántulas de 20 días de edad, los cuales fueron inyectados directamente con *Agrobacterium rhizogenes* ATCC13332 Los explantes tratados se cultivaron en medio Murashige y Skoog (MS) solidificado, libre de reguladores del crecimiento. **Resultados.** Se indujeron raíces pilosas en el 55.86% de los explantes foliares, lo cual representó el primer indicador visible de transformación genética exitosa. La inducción de callo a partir de raíces pilosas alcanzó un 100% de éxito en el medio MS2 (MS + 1.0 $mg\cdot L^{-1}$ de benciladenina [BA] + 0.1 $mg\cdot L^{-1}$ de ácido indolbutírico [IBA]). El análisis mediante (PCR) confirmó la presencia del gen rolB en el ADN genómico de los tejidos transformados. Se observó regeneración de brotes durante el mantenimiento de las raíces pilosas. La adición de ZnO NPs (25–300 µg/mL) al medio MS mejoró significativamente la inducción de raíces pilosas, alcanzando un 100 % en explantes foliares con 150 µg/mL. **Conclusiones.** Las nanopartículas de óxido de zinc resultaron eficaces para aumentar la frecuencia de transformación genética en *S. marianum*, lo que demuestra su potencial como herramienta nanobiotecnológica en la ingeniería genética vegetal.

---

[1] College of Education for Pure Sciences, University of Mosul, Iraq, ahellah@uomosul.edu.iq
[2] Department of Biology, College of Education for Pure Sciences, University of Mosul, Mosul, Iraq, *Corresponding author: ✉ dr.shifasalih@uomosul.edu.iq
[2] Department of Biology, College of Education for Pure Sciences, University of Mosul, Mosul, Iraq, dr.muthanna.j.m@uomosul.edu.iq

INTRODUCTION

Milk thistle (*Silybum marianum* L.) (Asteraceae) is native to the Mediterranean region and is now widespread worldwide (1-2). Historically, it has been used for the treatment of liver disorders, gallbladder ailments, and depression (3-4). Today, this plant is receiving growing attention for its cosmetic and medicinal applications. Its therapeutic potential is largely attributed to a flavonoid compound called silymarin, which possesses anti-inflammatory and antioxidant properties and is also used in treating drug-induced liver injury and viral hepatitis (5).

In addition to silymarin, *S. marianum* seeds contain a high oil content ranging from 20% to 30% (6). Various fractions of this plant are utilized in different industries, including oil extraction, animal feed, and renewable energy production (2,7).

Genetic transformation using *Agrobacterium rhizogenes* is an efficient and rapid technique applied in several plant species, including *Eruca sativa*, *Plukenetia volubilis*, and citrus (8-10). Upon infection by *A. rhizogenes*, hairy roots—neoplastic growths—form at the site of inoculation due to the integration of transfer DNA (T-DNA) from the root-inducing (Ri) plasmid into the plant genomic DNA (11). This T-DNA region carries the rolA, rolB, rolC, and rolD genes (12). Expression of these **rol** genes in host plant cells alters auxin homeostasis, which in turn promotes the proliferation of hairy roots at the wounded sites. Consequently, the initiation of hairy root cultures generally does not require phytohormone supplementation (11).

Among these genes, rolB plays a critical role in root induction and is associated with auxin sensitivity, often producing distinct phenotypes in transformed plants. This gene is also known to influence plant genetic pathways by stimulating meristematic cells and directing their development not only into roots but potentially into other tissues or organs (13). For decades, rolB has been considered essential in rooting processes (12) and is also a strong inducer of secondary metabolite production in transformed tissues (14). Moreover, it has been shown to modulate various physiological responses, including free radical scavenging and enhanced photosynthetic efficiency (13).

In recent years, nanotechnology has opened new avenues in both basic and applied plant sciences (15). Nanomaterials—defined as materials with dimensions between 1 and 100 nanometers—possess unique physicochemical properties due to molecular-level structural modulation, allowing rapid biological activity (16). Various nanoparticles (NPs), including those composed of zinc, gold, silver, magnesium, carbon, and copper, have demonstrated potential applications across medicine, cosmetics, agriculture, and environmental sciences (15).

It has been proposed that nanoparticles can enhance the efficiency of plant genetic transformation by facilitating the uptake and integration of exogenous DNA and by overcoming physical barriers such as the plant cell wall (17). This strategy can lead to the development of genetically modified plants with improved traits, including increased productivity and enhanced phytochemical profiles.

The present study aims to evaluate the response of *Silybum marianum* L. to genetic transformation mediated by *A. rhizogenes* and to establish stable hairy root cultures. In addition, this work explores whether the application of zinc oxide nanoparticles (ZnO NPs) can enhance the genetic transformation efficiency of this medicinal plant.

MATERIALS AND METHODS

Plant material

All experiments were conducted in the Plant Tissue Culture Laboratory, Department of Biology, College of Education for Pure Sciences at the University of Mosul, Iraq. Seeds of Silybum marianum L. were obtained from a local market in Mosul. For surface sterilization, seeds were immersed in 2% sodium hypochlorite (NaOCl) for 15 minutes, followed by three rinses with sterile distilled water. Sterilized seeds were cultured in 100 mL sterile glass jars containing 25 mL of hormone-free Murashige and Skoog (MS) medium (18). Cultures were kept in darkness at 25 ± 2 °C for the first three days and then transferred to a 16 h light / 8 h dark photoperiod.

Preparation of *A. rhizogenes* inoculum

A. rhizogenes strain ATCC13332 harboring the Ri plasmid was acquired from the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures). Inoculum preparation followed the method of Abdullah et al. (8). A single colony was cultured in 5 mL of nutrient broth (NB) and incubated in a shaker at 28 °C and 150 rpm for 72 h. Optical density (OD) at 600 nm was recorded, and the bacterial suspension with the highest OD (0.69) was used for inoculation *A. Rhizogenes*.

Direct injection of the explants

The direct injection method described by Nourozi et al. (19) was used. A total of 145 leaves, 90 cotyledons, and 57 stems from 20-day-old seedlings were injected with *A. rhizogenes* ATCC13332 using a 0.5 mL insulin syringe. Furthermore, leaves were specifically injected through the midribs. All explants were cultured on solid MS medium without plant growth regulators (PGRs). Additionally, Woody Plant (WP) medium (20) was used as a comparative treatment under the same conditions. Cultures were maintained in darkness at 25 ± 2 °C and monitored daily for the initiation of hairy roots.

Establishment of hairy roots cultures

Hairy roots successfully induced from different explants were excised and cultured on MS and WP media, both without PGRs and supplemented with 0.5 mg L^{-1} naphthalene acetic acid (NAA) (21). Cultures were subcultured every 10 days and maintained under the same conditions.

Callus initiation from hairy roots

Hairy root segments (~1–1.5 cm) were transferred to glass jars containing the following five different media types for callus induction: i) MS1: MS + 1.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA; ii) MS2: MS + 1.0 mg L^{-1} BA + 0.1 mg L^{-1} IBA; iii) MS3: MS + 3.0 mg L^{-1} BA + 0.5 mg L^{-1} IBA; iv)W1: WP + 0.5 mg L^{-1} NAA; v) W2: WP + 1.0 mg L^{-1} NAA. Twenty-four cultures were prepared for each medium type. All the jars were placed in the growth room at 25°C under a 16/8 h (light/dark) photoperiod. The callus initiated from hairy roots was maintained by subculturing on to the best medium for initiation every 15 days.

Detection of *rolB*

Genomic DNA was extracted from hairy roots, callus derived from hairy roots, regenerated shoots, and control (non-transformed) seedlings using a commercial kit (Promega Genomic DNA Purification Kit, USA). The presence of T-DNA was confirmed by PCR amplification of the rolB gene, following the primer set described by Hom-Utai et al. (22): Forward (F = 5'ATGGATCCCAAATTGCTATTCCTTCCACGA3'; Reverse (R) = 5'TTAGGCTTCTTTCTTCAGGTTTACTGCAGC3' (Target amplicon size: 580 bp). The PCR reaction mixture was prepared in a final volume of 24 µL using a commercial premix containing MgCl2, KCl, Tris-HCl, dNTPs, and Taq DNA polymerase, and was used to amplify the rolB gene. Amplification was carried out in a T100 Thermal Cycler (Bio-Rad, USA) under the following conditions: an initial denaturation at 95 °C for 10 minutes; 35 cycles of denaturation at 95 °C for 1 minute, annealing at 58 °C for 1 minute, and extension at 72 °C for 1 minute; followed by a final extension at 72 °C for 5 minutes (23). After the reaction, 5 µL of each PCR product was loaded onto a 2% agarose gel, and the amplified DNA bands were visualized using a high-sensitivity digital camera.

Effect of zinc oxide nanoparticles on hairy roots initiation

Explants (145 leaves, 90 cotyledons, and 57 stems) from 20-day-old seedlings were injected with *A. rhizogenes* ATCC13332 as described above. They were then transferred to MS medium supplemented with various concentrations of ZnO nanoparticles (25, 50, 100, 125, 150, 200, and 300 µg/mL). ZnO NPs (<100 nm particle size) were obtained from Sigma-Aldrich (UK). Cultures were incubated in darkness at 25 ± 2 °C. For callus induction, hairy roots were transferred to WP medium containing 1.0 mg L^{-1} NAA, also supplemented with ZnO NPs at concentrations of 50, 100, 150, and 200 µg/mL.

Statistical analysis

Since the data were parametric, analysis of variance (ANOVA) was used to compare means. Significant differences were identified using Duncan's Multiple Range Test (DMRT) at a 5% significance level [24]. A Completely Randomized Design with multiple replications was employed. All statistical analyses were performed using SPSS version 16 (IBM, New York, NY, USA).

RESULTS

Table 1 indicates significant differences between explants in induction rates and time to hairy root appearance. Leaf explants significantly outperformed others, with hairy roots appearing within 6 days **(Figure 1A)**. Cotyledons and stem sections both showed roots by 10 days **(Figure 1B, C)**, with no significant difference between them. No hairy roots appeared on the water-injected (control). The hairy roots were thin and white **(Figure 1D)**. The highest induction rate (55.86%) was observed on leaves.

Table 1. Hairy root induction in *Silybum marianum* explants following direct injection with *A. rhizogenes* ATCC13332 (no PGRs or ZnO NPs added)

Type explants	No. of Responsive / No of Inoculated explants	Hairy roots induction %	Period of hairy roots appearance (day)
Leaves	$81/145^{a}$	55.86	6
Cotyledonary Leaves	$22/90^{b}$	24.44	10
Stems	$21/57^{b}$	36.84	10
***Control**	$0/30^{c}$	0.0	0.0
P-value			0.00

*Explants inoculated with sterile distilled water. Values with different letters differ significantly ($p < 0.05$).

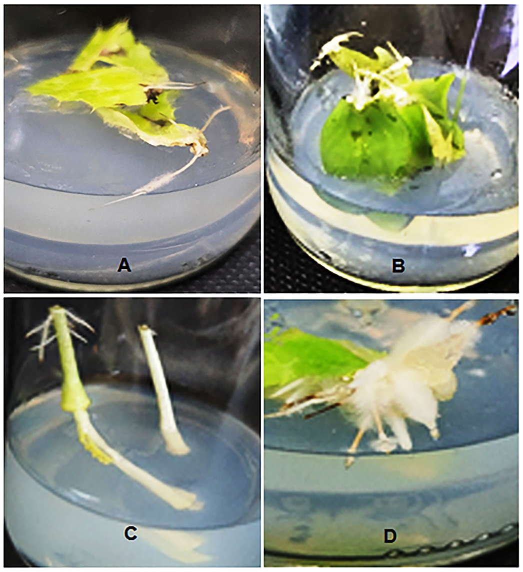


Figure 1: Direct injection of *S. marianum* explants by *A. rhizogenes* on MS medium. (**A**) Hairy roots on leaves. (**B**) Hairy roots on cotyledonary leaves. (**C**) Hairy roots on stems. (**D**) Culture of hairy roots.

Interestingly, hairy roots derived from leaf explants grown on MS medium supplemented with 0.5 mg L^{-1} NAA occasionally regenerated shoots **(Table 2)**. Regeneration initiated via callus formation, accompanied by the emergence of small leaf primordia (indicative of organogenesis) within one week of subculture **(Figure 2A)**, and shoots were fully developed within one month **(Figure 2B)**. However, all attempts to root these regenerated shoots failed.

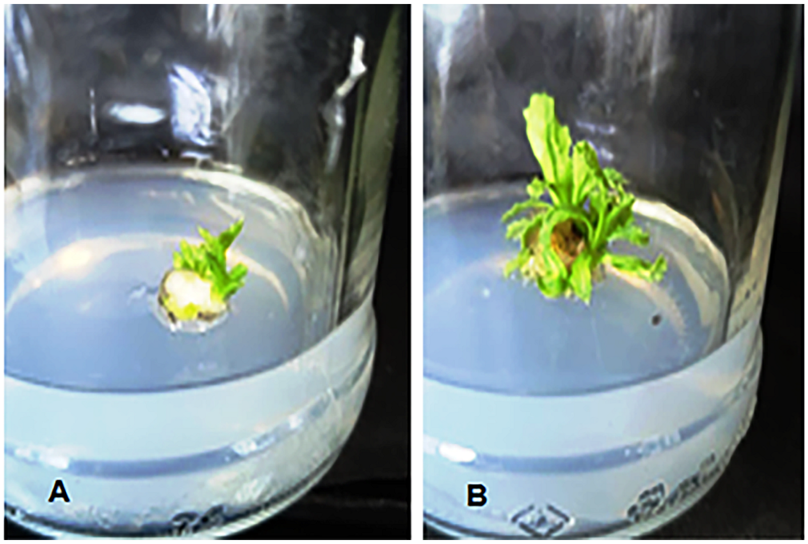


Figure 2: Shoot regeneration from *S. marianum* hairy roots (leaf explants) on MS + 0.5 mg/L NAA. (**A**) Shoots after one week. (**B**) Shoots after one month.

The ability of hairy roots to initiate callus varied by explant, origin, and cultured medium **(Table 2)**. For leaf-derived roots, on media MS2, MS3, and W2, all hairy root segments (6/6) formed callus (100%), significantly higher than others. For cotyledon roots, MS2, MS3, and W2 also outperformed others **(Table 2)**. For stem-derived roots, W1 and W2 were the best (6/6, 100%) versus only 2.75/6 on MS3 (45.4%). W2 medium was selected as the best medium for inducing and maintaining callus from hairy roots **(Figure 3A, B, C)**.

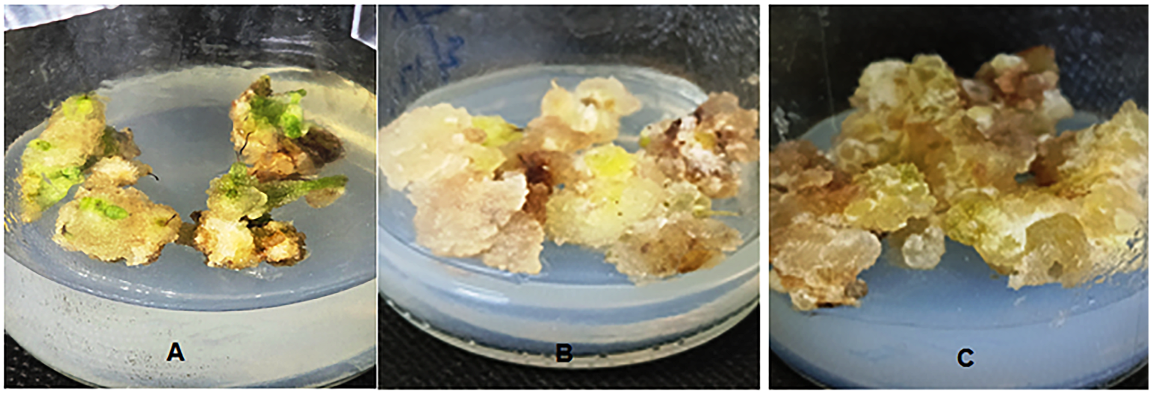


Figure 3: Callus induction from transformed hairy roots originated from different explants of *Silybum marianum* L. on WP + 1.0 mg L^{-1} NAA. (**A**) Callus of hairy roots originated from leaves. (**B**) Callus of hairy roots from cotyledonary leaves. (**C**) Callus of hairy roots from stems.

Meanwhile, *rolB* was not detected in the untransformed seedling samples, which served as the negative control **(Figure 4)**.

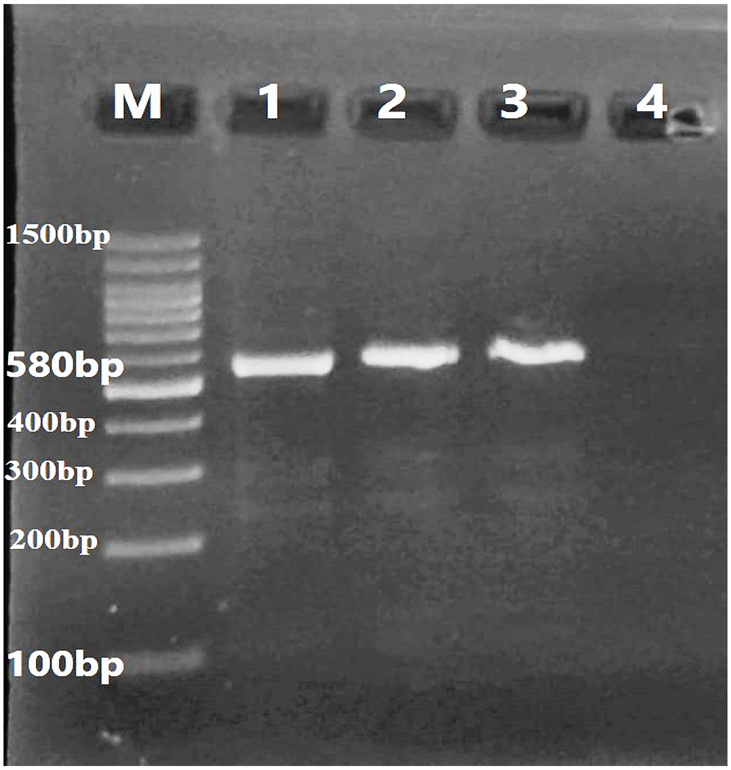


Figure 4. PCR analysis of genomic DNA from *Silybum marianum* L. tissues. **Lane M:** 1500-bp DNA ladder; **Lane 1:** DNA from hairy roots transformed by *A. rhizogenes* ATCC 13332; **Lane 2:** DNA from callus derived from hairy roots; **Lane 3:** DNA from genetically transformed shoots; **Lane 4:** DNA from seedlings (negative control).

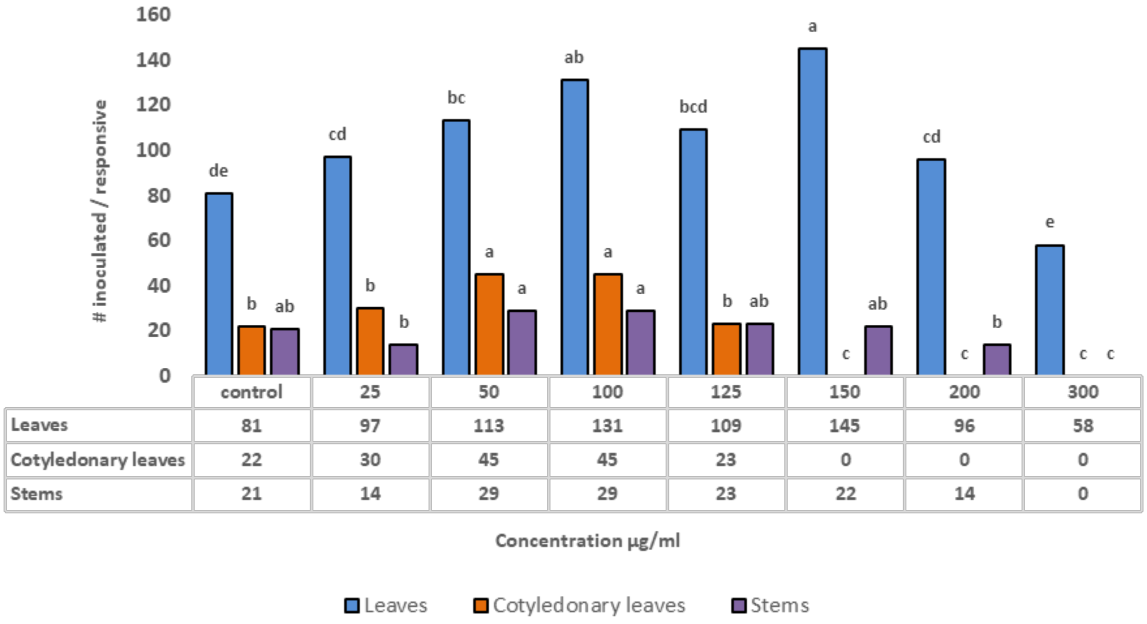
Table 2: Callus initiation from hairy roots of *Silybum marianum* on MS and WP media supplemented with different growth regulators

Media	**Number of hairy roots producing callus**			**Callus initiation frequency %**		
	Hairy roots of leaves	**Hairy roots of cotyledonary leaves**	**Hairy roots of stems**	**Hairy roots of leaves**	**Hairy roots of cotyledonary leaves**	**Hairy roots of stems**
MS1	5.25 ±0.50^{b}	4.50 ±1.29^{ab}	4.50±1.29^{b}	87.5	75	75
MS2	6.0 ±0.0^{a}	4.25 ±1.50^{bc}	6.0 ± 0.0^{a}	100	100	70.83
MS3	6.0 ±0.0^{a}	2.75 ±1.71^{c}	6.0±0.0^{a}	100	100	45.4
W1	4.50 ± 0.58^{c}	6.0 ±0.0^{a}	0.0 ±0.0^{c}	75	0	100
W2	6.0±0.0^{a}	5.25 ±0.96^{ab}	6.0 ±0.0^{a}	100	100	87.5
MSO control	0^{d}	0^{d}	0^{c}	0	0	0
WPO Control	0^{d}	0^{d}	0^{c}	0	0	0
P-value	0.00	0.025	0.00			

*The number of cultured hairy roots in each medium is 24. Values with different letters differ significantly ($p < 0.05$).

Treatment with ZnO nanoparticles significantly enhanced hairy root induction **(Figure 5)**. The highest induction rate (100%) was achieved in leaf explants cultured on MS medium with 150 µg mL-^{1} ZnO NPs. Other concentrations (50, 100, 125 µg mL-^{1}) also enhanced hairy root formation across explant types. In cotyledons, significant improvements were observed at 50 and 100 µg mL-^{1} compared to the control, while 150, 200, and 300 µg mL-^{1} yielded comparable results.

These results suggest that ZnO NPs are an effective elicitor of hairy root induction. However, the highest concentration tested (300 µg mL-^{1}) had a negative impact, reducing induction rates.



	control	25	50	100	125	150	200	300
Leaves	81	97	113	131	109	145	96	58
Cotyledonary leaves	22	30	45	45	23	0	0	0
Stems	21	14	29	29	23	22	14	0

Figure 5: Percentage induction of hairy root of different explants of *Silybum marianum* in MS medium fortified with various concentrations of ZnO NPs. Values with different letters differ significantly ($p < 0.05$).

DISCUSSIONS

Direct injection is considered a key transformation method and has been successfully applied in various plant species such as *Plumbago zeylanica* (25), *Calendula officinalis* (21), and *Eruca sativa* (8). The appearance of hairy roots serves as the first visible indicator of successful genetic transformation and T-DNA transfer from the Ri plasmid to the plant genome (26). The favorable response observed in leaf explants compared to other explants may be attributed to differences in endogenous hormone content among tissue types. Moreover, injections targeted the midrib area of the leaves, a region rich in active meristematic cells (27). Previous studies have confirmed that explant type and age significantly influence hairy root induction (28), as do the strain of *A. rhizogenes* (29-30) and the culture medium used (31).

Spontaneous shoot regeneration from hairy roots has been observed in other species as well. Abdullah et al. (8) reported spontaneous shoot formation during subculture and maintenance of hairy roots in *Eruca sativa* cultured on MS medium without growth regulators. Similarly, this phenomenon has been noted in hairy root cultures of the medicinal plant *Lopezia racemosa* (32). The regenerative capacity of hairy roots may be due to an alteration in endogenous hormone content following *Agrobacterium*-mediated transformation. It has been reported that *rolB* gene expression promotes the formation of new meristematic cells, which in turn trigger organogenesis (13). Additionally, shoot regeneration may be related to hormonal imbalance or nutrient depletion in the culture medium (33).

The success of callus formation from hairy roots could be associated with the expression of *rol* genes and auxin biosynthesis genes (*iaaM*, *iaaH*, *tmr*) located on the T-DNA, leading to auxin overproduction (34). Previous studies showed that transgenic cucumber roots containing the *iaaM* and *iaaH* genes were highly branched and thick, which was attributed to an overproduction of auxin (35). The detection of *rolB* in transformed hairy roots, regenerated shoots, and callus derived from hairy roots confirmed successful T-DNA integration. While the appearance of hairy roots is generally considered a preliminary indicator of successful genetic transformation with *A. rhizogenes* (36-37), *rol* gene detection is regarded as a definitive confirmation, as also supported by other studies (8, 38-39).

Nanoparticles are considered more efficient, functional, and prominent in their action compared to their macro-sized counterparts due to their nanoscale dimensions and increased surface area (15, 40). These properties enhance their effectiveness in various biological processes. The impact of nanoparticles on plant transformation has been demonstrated in several studies (17, 41). The significant effect of ZnO nanoparticles in enhancing hairy root induction observed in this study may be attributed to evidence that NPs facilitate horizontal gene transfer (HGT) between plant cells and bacteria (42). Additionally, ZnO NPs may act as elicitors that induce various physiological responses (40-41, 43). Their interaction with plant cell walls may also alter membrane permeability and cellular activity, as observed in *Trigonella foenum graecum* (41).

The results clearly indicated that low concentrations of ZnO nanoparticles significantly enhanced hairy root induction, whereas high concentrations (300 μg mL^{-1}) suppressed this process. Khan et al. similarly reported that low NP concentrations stimulate metabolic processes, while higher doses may be toxic due to accumulation in cellular organelles (44).

CONCLUSIONS

Successful genetic transformation of milk thistle (*Silybum marianum* L.) was achieved via *Agrobacterium rhizogenes* in this study. The application of zinc oxide nanoparticles (ZnO NPs) significantly enhanced the transformation frequency, particularly at 150 μg/mL in leaf explants. These findings may provide a useful platform for improving the production of secondary metabolites in this important medicinal plant.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. Katherine A. Steele from the School of Environment and Natural Sciences, Bangor University, UK, for proofreading the manuscript.

FUNDING

University of Mosul, College of Education for Pure Sciences, Iraq

ETHICAL CONSIDERATIONS

The current study is derived from a doctoral thesis registered in the Department of Biology and approved by the council of the College of Education for Pure Sciences according to administrative agreement No. 5969, 29-10-2023. It was performed in accordance with national and international standards of scientific laboratory safety.

Concerning the permission of collection the plant material used in the research, we used the seeds of *Silybum marianum* L. which were obtained from local markets in Mosul city, and identified by a specialist professor in plant taxonomy.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. **Abenavoli L**, **Capasso R**, **Milic N**, **Capasso F**. Milk thistle in liver diseases: past, present, future. *Phytother Res*. 2010 Oct;24(10):142332. https://doi.org/10.1002/ptr.3207 PubMed

2. **Marceddu R**, **Dinolfo L**, **Carrubba A**, **Sarno M**, **Di Miceli G**. Milk thistle (*Silybum marianum* L.) as a novel multipurpose crop for agriculture in marginal environments: A review. *Agronomy*. 2022 Mar;12(3):729. https://doi.org/10.3390/agronomy12030729

3. **Federico A**, **Dallio M**, **Loguercio C**. Silymarin/silybin and chronic liver disease: a marriage of many years. *Molecules*. 2017 Jan 24;22(2):191. https://doi.org/10.3390/molecules22020191

4. **Bijak M**. Silybin, a major bioactive component of milk thistle (*Silybum marianum* L. Gaernt.): chemistry, bioavailability, and metabolism. *Molecules*. 2017 Nov 10;22(11):1942. https://doi.org/10.3390/molecules22111942

5. **Fulvio F**, **Martinelli T**, **Paris R**. Selection and validation of reference genes for RTqPCR normalization in different tissues of milk thistle (*Silybum marianum*, Gaert.). *Gene*. 2021 Feb 5;768:145272. https://doi.org/10.1016/j.gene.2020.145272

6. **Martinelli T**, **Potenza E**, **Moschella A**, **Zaccheria F**, **Benedettelli S**, **Andrzejewska J**. Phenotypic evaluation of a milk thistle germplasm collection: fruit morphology and chemical composition. *Crop Sci*. 2016 Nov;56(6):316072. https://doi.org/10.2135/cropsci2016.03.0162

7. **Teleszko M**, **Haraf G**, **Zając A**, **Krzos G**. Milk thistle (*Silybum marianum* (L.) Gaertner) endosperm as an alternative protein source for a sustainable food system (SFS)—pilot studies. *Sustainability*. 2023 Oct 1;15(19):14411. https://doi.org/10.3390/su151914411

8. **Abdullah R**, **AlJirjees R**, **Salih S**. Effect of genetic transformation via *Agrobacterium rhizogenes* on the anatomical characteristics of *Eruca sativa* Mill. plants. Mesopotamia J Agric. 2023 Dec 1;51(4):1300. http://10.33899/mja.2023.143910.1280 Revista Académica Iraquí+1

9. Lin K, Lu LX, Pan BZ, Chai X, Fu QT, Geng XC, et al. *Agrobacterium rhizogenes*mediated hairy roots genetic transformation using Agrobacterium gel inoculation and RUBY reporter enables efficient gene function analysis in sacha inchi (*Plukenetia volubilis*). *Int J Mol Sci*. 2025 Mar 11;26(6):2496. https://doi.org/10.3390/ijms26062496

10. Wang M, Qin YY, Wei NN, Xue HY, Dai WS. Highly efficient *Agrobacterium rhizogenes*mediated hairy roots transformation in citrus seeds and its application in gene functional analysis. *Front Plant Sci*. 2023 Oct 31;14:1293374. https://doi.org/10.3389/fpls.2023.1293374

11. Cardarelli M, Mariotti D, Pomponi M, Spano L, Capone I, Costantino P. *Agrobacterium rhizogenes* TDNA genes capable of inducing hairy root phenotype. *Mol Gen Genet* (MGG). 1987 Oct;209(3):47580. https://doi.org/10.1007/BF00331152

12. Mauro ML, Bettini PP. *Agrobacterium rhizogenes* rolB oncogene: an intriguing player for many roles. *Plant Physiol Biochem*. 2021 Aug 1;165:108. https://doi.org/10.1016/j.plaphy.2021.04.037

13. Nilsson O, Olsson O. Getting to the roots: the role of the *Agrobacterium rhizogenes* rol genes in the formation of hairy roots. *Physiol Plant*. 1997 Jul;100(3):46373. https://doi.org/10.1111/j.1399-3054.1997.tb03050.x

14. Vasyutkina EA, Yugay YA, Grigorchuk VP, Grishchenko OV, Sorokina MR, Yaroshenko YL, Kudinova OD, Stepochkina VD, Bulgakov VP, Shkryl YN. Effect of stress signals and IbrolB/C overexpression on secondary metabolite biosynthesis in cell cultures of *Ipomoea batatas*. *Int J Mol Sci*. 2022 Dec 1;23(23):15100. https://doi.org/10.3390/ijms232315100

15. Findik F. Nanomaterials and their applications. *Period Eng Nat Sci*. 2021;9(3):6275. https://pen.ius.edu.ba/index.php/pen/article/view/828

16. Sabourian P, Yazdani G, Ashraf SS, Frounchi M, Mashayekhan S, Kiani S, Kakkar A. Effect of physicochemical properties of nanoparticles on their intracellular uptake. *Int J Mol Sci*. 2020;21(21):8019. https://doi.org/10.3390/ijms21218019

17. Lv Z, Jiang R, Chen J, Chen W. Nanoparticlemediated gene transformation strategies for plant genetic engineering. *Plant J*. 2020 Nov;104(4):88091. https://doi.org/10.1111/tpj.14973

18. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*. 1962 Jul 1;15(3):47397. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

19. Nourozi E, Hosseini B, Hassani A. Influences of various factors on hairy roots induction in *Agastache foeniculum* (Pursh) Kuntze. *Acta Agric Slov*. 2016 Apr 6;107(1):4554. https://doi.org/10.14720/aas.2016.107.1.05

20. Lloyd G, McCown B. Commerciallyfeasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoottip culture. *Combined Proceedings International Plant Propagators' Society*. (URL): https://www.pubhort.org/ipps/30/99.htm

21. AlAbasi I, AlMallah MK, Kassab BB. Engineering of high production of salicylic acid in transgenic hairy roots of *Calendula officinalis* L. by *Agrobacterium rhizogenes* ATCC 13332. *Afr J Biotechnol*. 2022 Dec 31;21(12):5718. https://academicjournals.org/journal/AJB/article-abstract/C99192C70154

22. **Hom-Utai S**, **Panichayupakaranant P**, **Wungsintaweekul J**, **Tansakul P**. Rhinacanthin production by four hairyroot lines of *Rhinacanthus nasutus* (L.) Kurz. *Planta Med*. 2009 Jul;75(9):PD23. https://kb.psu.ac.th/psukb/handle/2010/5910

23. **Alamholo M**, **Katayounchah RN**, **Soltani J**. Hyoscyamine and scopolamine production in induced callus and hairy roots of *Hyoscyamus niger*. *Academia J Biotech*. 2019 Dec;7(12):207215. https://doi.org/10.15413/ajb.2019.0114

24. **Duncan DB**. Multiple range and multiple F tests. *Biometrics*. 1955 Mar;11(1):142. https://doi.org/10.2307/3001478

25. **Basu A**, **Joshi RK**, **Jha S**. Genetic transformation of *Plumbago zeylanica* with *Agrobacterium rhizogenes* strain LBA 9402 and characterization of transformed root lines. *Plant Tissue Cult Biotechnol*. 2015 Jul;25(1):2135. https://doi.org/10.3329/ptcb.v25i1.24123

26. **Kısa D**, **Ceylan Y**, **İmamoğlu R**. Accumulation of phenolic compounds and expression of phenylpropanoid biosynthesisrelated genes in leaves of basil transformed with *A. rhizogenes* strains. *Prog Med Biol Plant*. 2023 May;29(5):62940. https://doi.org/10.1007/s12298-023-01320-w

27. **Khlifa HD**, **Klimek-Chodacka M**, **Baranski R**, **Combik M**, **Taha HS**. *A. rhizogenes*-mediated transformation of *Hypericum sinaicum* L. for the development of hairy roots containing hypericin. *Braz J Pharm Sci*. 2020 Nov 6;56:e18327. https://doi.org/10.1590/s2175-97902020000118327

28. **Amini S**, **Fattahi M**, **Nazemiyeh H**. Optimization of induction and hairy roots culture establishment in two mullein species, *Verbascum erianthum* and *Verbascum stachydiforme*. *Sci Rep*. 2024 Mar 7;14(1):5636. https://doi.org/10.1038/s41598-024-56331-8

29. **Sathasivam R**, **Choi M**, **Radhakrishnan R**, **Kwon H**, **Yoon J**, **Yang SH**, **Kim JK**, **Chung YS**, **Park SU**. Effects of various *Agrobacterium rhizogenes* strains on hairy root induction and analyses of primary and secondary metabolites in *Ocimum basilicum*. *Front Plant Sci*. 2022 Oct 17;13:983776. https://doi.org/10.3389/fpls.2022.983776

30. **Zhong Y**, **Zhou Z**, **Yin Z**, **Zhang L**, **Zhang Q**, **Xie Y**, **Chen J**. Effect of different *A. rhizogenes* strains on hairy root induction and analysis of metabolites in *Physalis peruviana* L. *J Plant Physiol*. 2025 Feb 1;305:154431. https://doi.org/10.1016/j.jplph.2025.154431

31. **Parizi AP**, **Farsi M**, **Nematzadeh GA**, **Mirshamsi A**. Impact of different culture media on hairy root growth of *Valeriana officinalis* L. *Acta Agric Slov*. 2014 Dec 4;103(2):299305. https://doi.org/10.14720/aas.2014.103.2.14

32. **Vargas-Morales N**, **Moreno-Anzúrez NE**, **Téllez-Román J**, **Perea-Arango I**, **Valencia-Díaz S**, **Leija-Salas A**, et al. Spontaneous regeneration of plantlets derived from hairy roots cultures of *Lopezia racemosa* and the cytotoxic activity of their organic extracts. *Plants*. 2022 Jan 6;11(2):150. https://doi.org/10.3390/plants11020150

33. **Altamura MM**. *Agrobacterium rhizogenes* rolB and rolD genes: regulation and involvement in plant development. *Plant Cell Tiss Organ Cult*. 2004 Apr;77:89101. https://doi.org/10.1023/B:TCGU.0000016609.22655.33

34. Hamill JD. Alterations in auxin and cytokinin metabolism of higher plants due to expression of specific genes from pathogenic bacteria: a review. *Funct Plant Biol*. 1993;20(5):40523. https://doi.org/10.1071/PP9930405

35. Amselem J, **Tepfer M**. Molecular basis for novel root phenotypes induced by *Agrobacterium rhizogenes* A4 on cucumber. *Plant Mol Biol*. 1992 Jun;19:42132. https://doi.org/10.1007/BF00023390

36. Sharma P, **Padh H**, **Shrivastava N**. Hairy root cultures: a suitable biological system for studying secondary metabolic pathways in plants. *Eng Life Sci*. 2013 Jan;13(1):6275. https://doi.org/10.1002/elsc.201200030

37. GutierrezValdes N, **Häkkinen ST**, **Lemasson C**, **Guillet M**, **OksmanCaldentey KM**, **Ritala A**, et al. Hairy root cultures—a versatile tool with multiple applications. *Front Plant Sci*. 2020 Mar 3;11:33. https://doi.org/10.3389/fpls.2020.00033

38. Arshad W, **Haq IU**, **Waheed MT**, **Mysore KS**, **Mirza B**. *Agrobacterium*-mediated transformation of tomato with *rolB* gene results in enhancement of fruit quality and foliar resistance against fungal pathogens. *PLoS One*. 2014 May 9;9(5):e96979. https://doi.org/10.1371/journal.pone.0096979

39. Kayani WK, **Palazon J**, **Cusidò RM**, **Mirza B**. The effect of *rol* genes on phytoecdysteroid biosynthesis in *Ajuga bracteosa* differs between transgenic plants and hairy roots. *RSC Adv*. 2016;6(27):227008. https://doi.org/10.1039/C6RA00250A

40. Karimi N, **Behbahani M**, **Dini G**, **Razmjou A**. Enhancing the secondary metabolite and anticancer activity of *Echinacea purpurea* callus extracts by treatment with biosynthesized ZnO nanoparticles. *Applied Nanoscience*. 2018 Nov 29;9(4):045009. https://doi.org/10.1007/s13204-018-0763-5

41. Tariverdizadeh N, **Mohebodini M**, **Chamani E**, **Ebadi A**. Iron and zinc oxide nanoparticles: an efficient elicitor to enhance trigonelline alkaloid production in hairy roots of fenugreek. *Ind Crops Prod*. 2021 Apr;162:113240. https://doi.org/10.1016/j.indcrop.2021.113240

42. Cui Y, **Zhao H**, **Zhang C**. Zinc oxide nanoparticles enhance plasmid transfer among growth-promoting endophytes in *Arabidopsis thaliana*. *Sci Total Environ*. 2024 Feb 25;913:169682. https://doi.org/10.1016/j.scitotenv.2023.169682

43. Javed R, **Yucesan B**, **Zia M**, **Gurel E**. Elicitation of secondary metabolites in callus cultures of *Stevia rebaudiana* grown under ZnO and CuO nanoparticle stress. *Sugar Tech*. 2018 Apr;20(2):194201. https://doi.org/10.1007/s12355-017-0539-1

44. Khan MA, **Raza A**, **Yousaf R**, **Ali H**, **Darwish H**. Impact of zinc oxide nanoparticles on biosynthesis of thymoquinone in cell cultures of *Nigella sativa*. *Plant Nano Biol*. 2024 Nov 1;10:100109. https://doi.org/10.1016/j.plana.2024.100109