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Plant regeneration from transformed tissues of Broccoli (*Brassica Oleracea var. Italica*) by *Agrobacterium rhizogenes* ATCC13332 and their retention of *rol*-Genes

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Abstract

This study was able to produce a transgenic plant of Broccoli, Brassica Oleracea var. Italica, by using the bacterial strain Agrobacterium rhizogenes ATCC13332 and its plasmids, which produced morphogenesis of genetically transformed hairy roots from stems and leaves. The better response of hairy roots formation was shown at leaves, which inoculated with both bacterial inoculum and their plasmids, with 81% and 85% frequency recorded respectively, as to frequency achieved of their induction from stems which was 41% and 50% respectively. Shoots were stimulated from the transformed hairy root cultures on MSO medium after four months of inoculation. These shoots were rooted, then, these plantlets were acclimatized, and transferred to the field. Polymerase chain reaction (PCR) of the amplified nucleic acids samples, using the primers of the rolC genes carried on the T-DNA segment, proved that the transgenic broccoli tissues retained these genes responsible of the formation of transgenic hairy roots that acquired different phenotypic characteristics from that of normal plants.

Keywords: Plant regeneration, transformed tissues of Broccoli (*Brassica Oleracea var. Italica*), *Agrobacterium rhizogenes* ATCC13332, retention of *rol*-Genes

INTRODUCTION:

The genus *Agrobacterium* was used to transfer T-DNA genes carried on pRi-DNA (Root inducing plasmid) or on pTi-DNA (Tumor inducing plasmid)

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responsible for the formation of hairy roots and crown gall tumours respectively in infected plants (Daspute *et al.*, 2019). These plasmids have succeeded in genetic modification of many plant species, such as rubber (Abdullah and Al-Mallah, 2011), sugar beet (AL-Mallah and Al-Nema, 2012), fenugreek (Al-Mahdawi, 2013), tomatoes (Al-Taie *et al.*, 2015), carrots (Al-Mallah and Muhammad, 2018), and cabbages (Al-Musayyib and Al-Mallah, 2019).

The hairy root disease is caused by the presence of four types of genes called *rol* genes (root locus), and they are *rolA*, *rolB*, *rolC*, *rolD*, and each of them has a function that appears when it is expressed, (Pavlova *et al.*, 2014). Broccoli plants (*Brassica oleracea var. italic*) belong to the cruciferous family *Brassicaceae*, which includes many plants, including kale, cauliflower, broccoli, and mustard (Shubha *et al.*, 2020). Broccoli is one of the important vegetables characterized by its high content of vitamins (A, B1, B2, B5, B6 and C) and many minerals (calcium, manganese, potassium, sodium). It is also rich in sulforaphane which can inhibit the growth of cancer cells and prevent blindness (Owis, 2015; Yagishita, *et al.*, 2019), and it is one of the most important antioxidants. In addition to that, it contains Indol-3-carbinol, an antioxidant chemical compound that prevents breast and colon cancer and enhances liver functions (Owis, 2015). It also has important nutritional and therapeutic benefits, including its role in regulating the level of sugar in the blood, and reducing the level of cholesterol in it, and it contributes to the bone building as well (Jang *et al.*, 2015). Broccoli is grown for its inflorescences that vary in colour from green to greenish-violet and are eaten in the phase of vegetative flowering buds with their thick silver stalks (Miraj, 2016).

The current study aims to include the *rolC* genes and the production of transgenic broccoli plants by direct injection and to find the extent to which their transformed tissues retain the *rolC* genes.

MATERIALS AND METHODS:

* Plant material:

Broccoli (*Brassica Oleracea var. Italica*) seeds was obtained from the local markets of Erbil governorate – Iraq, sterilized by washing with tap water for 30 min., then submerged in ethyl alcohol solution (70%) for one min., then immersed in sodium hypochlorite solution NaOCl (commercial bleach solution at a concentration of 6%) at a ratio of 1 unite volume of sterile substance: 1 unit volume of sterile water for ten minutes, then washed with sterile water three times, for two minutes each time, to remove traces of the sterile substance (Pavlovic *et al.*, 2010). The sterile seeds were sown, after drying by placing them on a solid surface of 20 ml of MSO medium (Murashige and Skoog, 1962), free of growth regulators, in a 100 ml glass vial at a rate of four

seeds/ vial. The samples were kept in the culture room under 16 h light/8 h darkness at 25 ± 2°C conditions.

Bacterial Strain and its Plasmids

Wiled strain *A. rhizogenes ATCC 13332* containing Ri plasmid which forms the hairy roots was supplied from the Leibniz Institute for Microorganisms and Cell Cultures Ltd., Germany (DSMz), and it was maintained periodically on Nutrient Agar solid medium. pRi plasmids were isolated from bacteria using a Plasmid Extraction kit.

Inoculation of Explants

Sterilized and excised stems cuttings of 15-day-old seedlings of 2-3 cm in length were directly inoculated using a minute needle (ICC Insulin Syringe U-100-29 G1/2-0.3314M); its end was immersed in the bacterial inoculum and its plasmids, and the stems were pricked at four locations. Leaves were also pricked in two or more locations of the lower surface of the Midvein, and also at the base of the leaf, the inoculated sections were planted in an upright position in solid MS, WPO medium in 100 ml vials containing 25 ml of these media at an average of 2 pieces/vial. The samples were preserved under culture room conditions at 25°C in complete darkness (AL- Nema and AL-Mallah, 2016).

Establishment of Genetically Transformed Hairy Roots Cultures Cured from Bacteria

Tufts of induced hairy roots were cut off from leaves and stems as a result of bacterial infection, and transferred to Petri dishes containing solid MSO and WPO medium supplemented with graded concentrations of cefotaxime 100, 200, 250, 300 mg/L and kept on each medium for 20 days and then maintained on MSO and WPO media supplemented with gradually decreasing concentrations to heal roots from bacteria and to maintain them periodically every 20 days (AL- Nema and AL- Mallah, 2016). They were kept under the aforementioned growth room conditions.

*** Formation of Differentiated Vegetative Branches from Hairy Roots; their Rooting and Acclimatization**

Shoots were formed from hairy roots after four months of periodic maintenance, and after 20 days of formation, shoots with a length of approximately 2.0 cm were separated from the transgenic hairy roots using a sharp sterile scalpel and were planted individually in 100 ml vials containing 20 ml of MSO- medium free from growth regulators, and MS medium containing 20 g sucrose and supplemented with IBA 1.0 and 2.0 mg/L, all samples were kept in the culture room (Pavlovic *et al.*, 2010). All plants

resulting from differentiation of hairy roots with appropriate root groups were taken from their media and well washed from the remnants of the medium with distilled water, and planted in plastic containers perforated from the bottom containing a mixture of sterilized soil with peat moss (1:1 V/V), and the plants were covered with perforated nylon bags for two days, and then these bags were removed and the samples were kept in the culture room at 25 ± 2°C and under light lighting while maintaining high humidity to avoid drying out.

Genetic Analysis

DNA was isolated from hairy roots, normal roots, seed plants, and transgenic plants using isolation and purification solutions (Plant Genomic DNA Extraction Mini Kit, Favor gen, Taiwan). DNA isolated from plant tissues was used in the polymerase chain reaction (Alamholo *et al.*, 2019) to detect the transfer of *rol C* genes carried on the T-DNA segment transferred from plasmids of the bacterial strain to the genome of plant tissues and their integration into it with the help of specific primers.

F = 5' CATTAGCCGATTGCAAACTTG3' (Hom - utai, 2009).
R= 5' ATGGCTGAAGACCTG3'

At the end of the reaction time, 3 microliters of each were taken and put in pits of double agarose gel of 2% with Gel Red dye, and the bundles were photographed via a digital camera.

RESULTS

The results of inoculation of stem cuttings excised from sterile seedlings of Broccoli, and in the midvein and leaf base by injecting them directly with *Agrobacterium rhizogenes ATCC13332* strain and its plasmids and instilling them in WPO solid medium, showed that the inoculation was tolerated and they retained viability and the emergence of the hairy root of these samples was varied (Table 1). The results also showed that the best medium for root induction was WPO medium.

Results (Table 1) express the readiness and superiority of true leaves over stem cuttings in the induction of hairy roots with bacterial inoculation of 72 hours incubation period (O.D = 1.6) and the lower induction rate of hairy roots upon direct injection of other densities.

Table (1): Induction of hairy roots on stem cuttings of broccoli seedlings *B. oleracea var.* Italica and on true leaves with *A. rhizogenes ATCC13332* grown on WP solid medium.

Plant Part	Inoculation Density (O.D)	Inoculated/ Respondent Number	Induction Rate %	Induction Period (Day)
Stem Cuttings	0.3	23 /75	30.6	16
	0.7	33 /90	36.6	16
	1.6	37 /90	41.1	15
	2.4	15 /75	20.0	18
	* Control	0 /25	0.0	0.0
Leaves	0.3	41 /75	54.6	10
	0.7	64 /90	71.1	7
	1.6	73 /90	81.1	7
	2.4	20 /75	26.6	9
	* Control	0 /25	0.0	0.0

* Control samples inoculated with sterile distilled water.

Generally, hairy roots formed first in the injection locations, and the response of the leaves was early, especially at the two densities (0.7 and 1.6) better than the inoculated stem cuttings at the same conditions, as the hairy roots emerged in the form of small, bright white tufts of dense hairs, in addition to their emergence in non-inoculated sites in the true leaves. They appeared on both sides of the leaf in the midvein area and at the base of the leaf (Fig. 1, A, B, C), and the inoculation of the stem cuttings resulted in the emergence of bright roots in the form of tufts at the injection sites and at sites that were not inoculated, with a period similar to the period of their emergence from the inoculated sites, later developed into a dense group of hairy roots, with a white color (Fig. 1, D, E).

Fig. 1: Emergence of hairy roots on the plant parts of broccoli plant *B. oleracea var.* Italica inoculated by direct injection with *Agrobacterium rhizogenes ATCC13332* or its developing plasmids and the formation of vegetative branches directly from the hairy roots in WP medium

A: Hairy roots formed (marked part) on both sides of the leaf at the injection sites.
B: Hairy root morphogenesis (marked part), non-inoculated sites.
C: hairy root morphogenesis (marked part) at the base of the leaf.

D: induced hairy roots (marked part) on stem cuttings at inoculation areas.
E: Hairy roots (marked part) on stem cuttings in areas not inoculated with bacteria.
F: Appearance of true leaves (marked part) from hairy root culture on MSO medium.
G: The elongation of the shoots increases and their growth development 10 days after the appearance of the true leaves.
H: Increase of the number of shoots and their rapid growth and development on the same medium.

Direct morphogenesis of vegetative branches from hairy roots:

One of the prominent results in this study is the appearance of green micro-structures in most of the genetically transformed hairy root cultures when they continue to be periodically maintained on MSO medium, and after four months of maintenance, they develop into true leaves (Fig. 1, F), and with continuous maintenance, it unfolded into vegetative branches (Fig. 1, G); 2 - 13 branches/culture of the hairy root. These branches were characterized by their elongation and rapid growth (Fig. 1, H). The total number of branches reached 60 branches / 20 cultures of the hairy roots.

Rooting and acclimatizing transgenic Plants:

The results showed the viability of the differentiated branches of the transgenic hairy roots and the ease of rooting by 100%, taking 7-9 days (Fig. 2, A). The formed roots were characterized by their rapid growth; their number was between 5-7 roots and of varying lengths (Fig. 2, B). After that, the formed plants were transferred at the age of 20 days to a mixture of soil and sterilized peat moss for acclimatizing (Fig. 2, C) and when following their growth, it was noticed that their branches were short (Fig. 2, D) and their leaves were wrinkled (Fig. 2, E).

Fig. 2: Morphogenesis of transgenic broccoli plants *B. oleracea var. Italica* by directly emerging from hairy roots induced by *A. rhizogenes ATCC13332* bacteria

A: Rooting of vegetative branches (marked part) on solid MSO medium.
B: Note the difference in the number and length of roots arising from the vegetative branches resulting from the hairy roots and their callus.
C: Transplantation and acclimatization of transgenic plants in soil and peat moss mixture.
D: Transgenic broccoli plants of 45 days old grown in soil and peat moss mixture. Note their short branches.
E: Note leaf curl in transgenic broccoli plants (The marked part).
F: Seed plants of 50 days old.

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The results also showed the response of the broccoli plant to direct injection of Ri plasmids isolated from *A. rhizogenes ATCC13332* bacteria and the efficiency of inducing hairy roots from stem cuttings and true leaves. Leaves were superior to stem cuttings in their response (Table 2).

Table (2): Induction of hairy roots on stem cuttings and true leaves of broccoli seedlings, *Brassica oleracea var.* Italica inoculated with Ri plasmids of *A. rhizogenes ATCC13332* by direct injection.

Plant Part	Inoculated/ Respondent Number	Induction Rate %	Time for hairy roots formation (day)
Leaves	85 /100	85.0	7
Stems	38 /75	50.0	12
*** Control**	0 /25	0.0	0.0

* Plant parts inoculated with sterile distilled water.

Induction of hairy roots on stem cuttings inoculated with Ri plasmid was also observed. The results showed that the plant parts responded better to direct injection with pRi plasmids than they responded to direct injection with bacteria, as hair root tufts were formed 7-12 days after injecting the plant parts with pRi plasmid, in addition to the absence of bacterial contamination that was present when the bacterial suspension was injected. The rate of induction increased from 81-85% on true leaves, while the rate of induction in stem cuttings increased from 41-50%.

Concentration and Purity of Isolated DNA:

The data showed a discrepancy in the concentration of DNA isolated from hairy roots and the plants genetically transformed by *A. rhizogenes ATCC13332* bacteria compared to its concentration in normal roots and broccoli seed plants (Table: 3). It was also observed that the purity of the DNA isolated from all tissues was appropriate for the parts of the polymerase chain replication in the later step in terms of the separation of the DNA bundles in the gel layer and in the presence of the volumetric marker.

Table (3): Concentration and purity of DNA isolated from tissues of broccoli *B. oleracea var.* Italica genetically transformed by *A. rhizogenes ATCC13332* bacteria and from induced hairy roots, hairy root callus, stem callus and seed plants

Source of isolated DNA (mg/l^{-1})	Concentration (Ng/Ml^{-1})	Purity
Normal roots	122.9	1.8
Transgenic hairy roots	202.9	1.7
Transgenic plants	205.5	1.6
Seed plants	124.2	1.7

Retention of *rolC* genes by Broccoli genetically transformed by *A. rhizogenes ATCC13332* bacteria

The results of gel electrophoresis of the sPCR reaction showed the separation of bundles of duplicated DNA of hairy roots and transgenic plants, and their molecular size was equal to the molecular size of the specific primers of *rol* C genes in the gel layer (Fig. 2), noting the absence of separation of such bundles of amplified DNA isolated from normal roots and seed plants. This result indicates the transmission of the T-DNA segment from bacteria and its integration with the genome of genetically transformed broccoli hairy root tissues with the group of *rol*-genes.

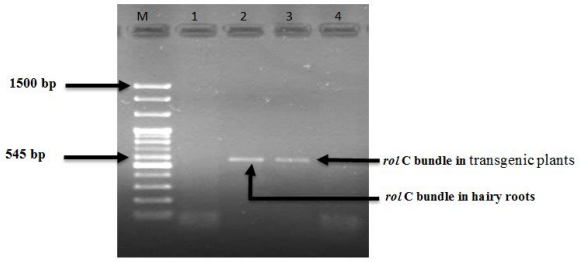

Fig. 2: Transfer of *rol C* genes in an amplified DNA sample isolated from hairy roots and broccoli plants genetically transformed by *A. rhizogenes ATCC13332* bacteria by electrophoresis in 1.5% agarose.

Path (M): volumetric marker λDNA.
Path (1): DNA amplified and isolated from the roots of seed plants.
Path (2): DNA amplified and isolated from hairy roots genetically transformed by *A. rhizogenes ATCC13332*.
Path (3): DNA amplified and isolated from plants genetically transformed by the bacterial vector *A. rhizogenes*.
Path 4: DNA amplified and extracted from seed plants.

DISCUSSION:

In the current study, the use of the strain *ATCC13332 A. rhizogenes* to bring about a genetic transformation in broccoli plants indicates that this wild type strain did not undergo any changes in its genetic material, including genetic markers, i.e., it is a naturally engineered strain (Geng *et al.*, 2012); it did not

differ from the genetically modified strains with *A. Rhizogenes* bacteria, either by direct injection or by using its plasmid (karthikeyan, *et al.*, 2007). The success of the genetic transformation in this plant indicates the compatibility between the plant type and the bacterial strain and its plasmids, and the hairy roots are the first signs of genetic transformation from stem cuttings and leaves removed from young seedlings of broccoli (Kowalczyk *et al.*, 2018). The main reason for the occurrence of genetic transformation is due to the wounding of plant parts and their secretion of phenolic compounds such as *acetosyringone* and sugar molecules that have a role in activating the *vir* gene group and are responsible for causing genetic transformation (Gelvin, 2009). The superiority of leaf tissues over stem tissues of the broccoli plant may be attributed to the nature of the hollow stems, which weakens their ability to tolerate virulence genes. The plant part used is one of the factors affecting the emergence of hairy roots (Samadi *et al.*, 2012). The direct injection method is one of the important methods used in many studies, which have proven its efficacy and excellence in generating genetic transformation (Al-Nema and ALMallah, 2016).

The emergence of hairy roots in this study also may explain the occurrence of physiological and morphological disorders as a result of gene expression of T-DNA genes, including *iaaH* and *iaaM* genes that contribute to encoding the construction of cytokines and auxins, which led to a disturbance in the endogenous hormones that stimulated hairy root emergence (Chattopadhyay *et al.*, 2011), in addition to the formation of unusual amino acids (opines) which bacteria use as a source of carbon (Sharma *et al.*, 2019). Also, the superiority of genetic transformation rates when directly injected with Ri plasmids may be attributed to tissue behavior, type, genetic line and cell number in the plant part inoculated with the plasmid (Niazian *et al.*, 2017). The high ability of genetically transformed hairy roots to grow in MS medium devoid of growth regulators and their direct formation of vegetative branches after periodic maintenance of root cultures may be due to their possession of a segment of T-DNA that guarantees to obtain genetically transformed plants. The success achieved in this study in obtaining transgenic broccoli plant directly from the transgenic hairy roots despite the long period of time, is an important step in the transgenesis system and it occurs due to the effect of the *rol* genes present on the T-DNA segment as mentioned earlier (One and Tian, 2011). This is consistent with (Zargis, 2021) in obtaining *arabidopsis* plants directly in the hairy roots induced by the same wild strain.

The increase in the concentration of DNA in the tissues of hairy roots and transgenic plants, than the concentration of the rest of the extracts proves the success of the transfer of T-DNA genes carried on Ri plasmids into the tissues of broccoli plants and their integration with the genetic repertoire,

and thus led to an increase in the speed of cell divisions, and then an increase in the construction of amino acids as well as proteins (Balasubramanian *et al.*, 2018). The presence of *rolC* is indicated by the use of the polymerase chain reaction (PCR) technology, to which attributed the development of vegetative branches and the formation of transgenic plants (Zupan *et al.*, 2000).

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