Malaysian Journal of Microbiology, Vol 20(5) 2024, pp. 630-635
DOI: http://dx.doi.org/10.21161/mjm.230241

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Efficiency of direct injection and co-cultivation methods in genetic transformation of oilseed rape (*Brassica napus* L.) seedlings leaves by *Agrobacterium rhizogenes* ATCC15834

Zaid Tahseen Qaddawi[1]*, Safwan Jasim Sultan[2] and Amjad Abdul-Hadi Mohammed[1]

[1]Biology Department, College of Science, University of Mosul, Mosul, Iraq.
[2]College of Dentistry, Al-Noor University, Ninavah, Iraq.
Email: zaid.qaddawi@uomosul.edu.iq

Received 21 March 2023; Received in revised form 26 August 2023; Accepted 12 July 2024

ABSTRACT

Aims: The study was aimed to investigate the efficiency of direct and co-cultivation methods in the genetic transformation of *Brassica napus* at the molecular level.
Methodology and results: The seedlings' leaves were prepared from explants parts of oilseed rape and inoculated with a culture of *A. rhizogenes* ATCC15834 using direct injection and co-cultivation methods. Cultures of hairy roots were created, then were aggregated and placed on a solid MS medium and sub-cultured by transferring them 3-4 times on an MS solid medium containing graded concentrations (100, 200 and 250 mg/L) of cefotaxime until obtaining hairy root cultures free of bacteria. Moreover, the DNA was extracted and amplified from each type of hairy root by polymerase chain reaction (PCR). The results show that the process of the inoculation of the leaves by direct injection with the *Agrobacterium rhizogenes* ATCC15834 suspension at the density of 1.151 × 10^8 cells/mL was the most efficient way for the formation of hairy roots compared with the other densities after 30 days with 80.64% ratio and of 2.72 roots/segment average. Additionally, hairy roots were also formed on oilseed rape leaves by incubating them with co-cultivation and bacterial *A. rhizogenes* ATCC15834 at a density of 1.151 × 10^8 cells/mL for 4 h, which shows their superiority from the other incubation periods, recording 44.44% and 2.55 average. Gel electrophoresis results demonstrate that one band was amplified by PCR with a molecular weight of 590 bp, identical to the estimated size of *rol*C gene recovered from the genomic DNA of hairy roots using specific primers.
Conclusion, significance and impact of study: It is concluded that hairy roots can be produced from oilseed rape *B. napus* L. seedling leaves using direct injection and co-cultivation methods by *A. rhizogenes* ATCC15834.

Keywords: A. rhizogenes ATCC15834, *Brassica napus*, co-cultivation, direct injection

INTRODUCTION

The most often used technique for introducing genes into plants is genetic transformation mediated by an *Agrobacterium*. This technique is more affordable and easier compared to the majority of direct gene transfer techniques. It facilitates the incorporation of genes of interest into the plant genome and minimizes its rearrangement. All *A. rhizogenes* strains are characterized as Gram-negative soil bacteria with a rod form containing a large root-inducing (Ri) plasmid (Rana *et al*., 2017). Infection with *A. rhizogenes* produces hairy roots, which appear as a swarm of little, projecting roots resembling fine hairs. A specific transfer DNA (T-DNA) from the Ri plasmid helps a collection of genes (encoding enzymes involved in generating auxin and cytokinin) integrate into the genome (Fan *et al*., 2020).

Numerous characteristics of hairy-root cultures have encouraged the use of this technique in plant biotechnology applications. The genetic stability of these roots is another characteristic of them. Hairy roots have an advantage over plant cell suspension cultures in that they can produce foreign proteins and valuable secondary metabolites on a continuous basis. This is due to their ease of maintenance, quick growth, quick doubling time and capacity to synthesize a variety of chemical compounds and proteins (Cardillo *et al*., 2016; Korde *et al*., 2016).

Brassica napus L. (oilseed rape, rapeseed) is well-known as “Colza” in Iran, this plant belonging to the family Cruciferae, is one of the most significant oil crops, producing 15% of the world's edible oil (Dai *et al*., 2020).

Oilseed rape has been genetically altered using a variety of techniques, including electroporation, PEG-

*Corresponding author

ISSN (print): 1823-8262, ISSN (online): 2231-7538

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mediated DNA uptake, microspore transfection, *Agrobacterium*-mediated transformation and particle bombardment. *Agrobacterium*-mediated transformation is the most widely used, trustworthy and efficient of these technologies (Zhang *et al*., 2020).

In the field of oilseed rape plants' genetic transformation, a number of studies were conducted to find an efficient protocol and improve the quality of the plant if the genetically modified oilseed rape successfully formed hairy roots by using two distinct transformation techniques to produce transgenic oilseed rape plants, In the first technique, hairy root growth was induced at the cotyledonary nodes using a binary *A. rhizogenes* system made up of p Ri1855 and p Bin19 plasmids. The second method involved transforming the inflorescence stalks of various rape kinds using *A. tumefaciens* binary system (Boulter *et al*., 1990). Furthermore, utilizing wild-type *A. rhizogenes* help to examine transformation as a biotechnological technique in breeding for more branching and shorter oilseed rape. The number of branches grew noticeably in the second generation of converted oilseed rape, rising by 49% when compared to rol+/aux+ plants with WT, from 7.7 ± 0.4 to 11.5 ± 1.9. At the beginning of flowering, rol+/aux+ plants' apical height decreased by 25%, from 81.3 ± 1.9 cm to 62.4 ± 6.7 cm (Hegelund *et al*., 2018). This study aimed to investigate the efficiency of direct and co-cultivation methods in the genetic transformation of *Brassica napus* at the molecular level.

MATERIALS AND METHODS

Preparation of explants parts

Brassica napus L. seeds were surface sterilized by immersing them in the 70% ethyl alcohol solution with continuous stirring for 2 min, then submerged in 3% sodium hypochlorite solution for 10 min. Seeds were washed with sterile distilled water three times for 1 min (Bates *et al*., 2017). Five sterile seeds were put into each flask along with 30 mL of solid MS media (Murashige and Skoog, 1962). The samples were kept in the incubator room at 22 ± 2 °C in darkness. After the seedlings emerged, they were moved to light conditions of 16 h of light and 8 h of darkness with a light density of 1500 lux.

Inoculation of seedlings leaves with *A. rhizogenes* ATCC15834 by direct injection method

15-day-old oilseed rape seedlings leaves have part of the petiole inoculated using the direct injection method with *A. rhizogenes* inoculums after 24, 48 and 72 h age at density 1.035, 1.151, 0.930 × 10^8 cells mL^{-1}, respectively into the middle vein of the upper surface (Al-Mallah and Mohamed, 2012). The samples were cultured on the surface of 30 mL of MS solid medium at a rate of 2-3 pieces/flask and preserved in an incubator with a temperature of 22 ± 2 °C and 400 lux light intensity.

Inoculation of seedlings leaves with *A. rhizogenes* ATCC15834 by co-cultivation method

A group of complete leaves were incubated individually in the bacterial inoculum at a density 1.151 × 10^8 cells/mL for incubation periods 2, 4 and 6 h individually. Samples were washed with sterile water for 2 min and (Al-Mallah and Mohamed, 2012), then were put on MS medium supplemented with 250 mg/L of cefotaxime and incubated under low light conditions of 200 lux at 24 °C and followed.

Successive transfer of hairy root cultures on antibiotic-supported media

0.5 g of hairy roots formed on leaves that were injected by direct injection or co-cultivation with bacteria were excised and transferred to a sterile MS media separately. For eliminate bacteria, the culture of hairy roots was transferred 3-4 times on MS solid media containing graded concentrations of cefotaxime (100, 200 and 250 mg/L). The period between one transfer and another was three weeks (Mohammed and Masyab, 2020). The culture was preserved under the same conditions indicated above.

Proofness of hairy root genetic transformation by PCR

Plant genomic DNA was isolated from hairy roots grow for 30 days on MS media that were produced by direct injection and co-cultivation (with *A. rhizogenes* ATCC 15834). In addition, plant genomic DNA was isolated from 20 days old seedling roots as a control. DNA was isolated using a favorgen DNA extraction kit (Weigand *et al*., 1993). Plant genomic DNA was amplified by PCR using the specific primers rolC-F (5´-CATTAGCCGATTGCAAACTTG-3´) and rolC-R (5´-ATGGCTGAAGACGACCTG-3´) designed by Panda *et al*. (2017). The conditions used for amplification were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles consisting of a denaturation cycle at 94 °C for 1 min, an annealing cycle at 62 °C for 1.5 min and an extension step at 72 °C for 2 min. Finally, the PCR ended by a final extension cycle at 72 °C for 10 min. The amplification products were run on 1% agarose gel for 45 min under 80V electric current. PCR products were stained using Safe Red strain (Safe Red Stain Dye, Korea).

RESULTS AND DISCUSSION

Production of hairy roots from oilseed rape leaves inoculated by *A. rhizogenes* ATCC15834 using:

Direct injection method

Leaves that were inoculated by the direct injection with varying densities (1.035, 1.151, 0.930 × 10^8 cell/mL) of the bacterial inoculum revealed the stimulation of hairy

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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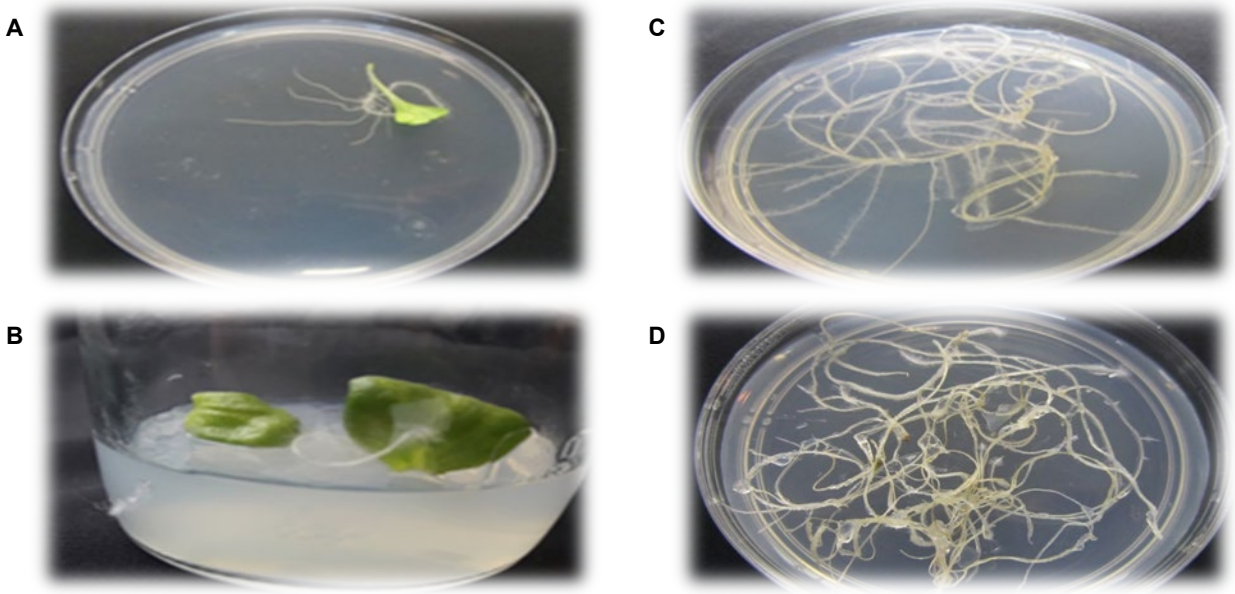

Figure 1: Hairy roots production on the leaves of oilseed rape (*B. napus* L.) plants seedlings inoculated by *A. rhizogenes* ATCC15834 by direct injection and its cultures. A: Hairy roots formed on leaves inoculated with density (1.151 × 10^8 cell/mL); B: Hairy roots formed on leaves inoculated with density (0.930 × 10^8 cell/mL); C: Culture of hairy roots developed from A; D: Culture of hairy roots developed from B.

Table 1: Production of hairy roots from oilseed rape (*B. napus* L.) seedlings leaves inoculated with *A. rhizogenes* ATCC15834 by direct injection and grown in MS solid medium for 30 days.

Bacterial inoculum density (×10^8 cell/mL)	No. of leaves inoculated/ responsive	Total no. of roots formed	Hairy roots production (%)	Average root lengths (cm)	Average of hairy roots/segment
1.035	29/18	35	62.06	0.24	1.7
1.151	31/25	68	80.64	0.32	2.72
0.930	34/22	42	64.70	0.5	1.90
Control*	25/0	0	0	0	0

*Control sample inoculated with distilled water.

Table 2: Production of hairy roots from oilseed rape (*B. napus* L.) seedlings leaves inoculated with *A. rhizogenes* ATCC15834 by co-cultivation and grown on solid MS medium after 30 days.

Bacterial inoculum density (×10^8 cell/mL)	Incubation time (hour)	No. of leaves inoculated/ responsive	Total no. of roots formed	Hairy roots production (%)	Average root lengths (cm)	Average of hairy roots/segment
1.151	2	55/22	43	40	3.5	1.95
	4	45/20	51	44.44	2	2.55
	6	50/18	30	36	3	1.66
Control*	for all periods	25/0	0	0	0	0

*Control sample inoculated with distilled water.

roots formation at the inoculation site after varying periods of time (Table 1).

The data showed that the density of 1.151 × 10^8 cells/mL was the most efficient in forming hairy roots compared to other densities after 30 days following direct injection, with a ratio of 80.64%. The other densities of the bacterial inoculum stimulated the hairy roots to different degrees.

The hairy roots are characterized by their white color and rapid growth (Figure 1A and 1B).

The variation in the response of leave pieces when inoculated with different densities of bacteria and the formation of hairy roots may be due to the effect of the density of the inoculum used, as well as the variation in the number of plant cells that responded to the pollination process by direct injection, which results in a variation in

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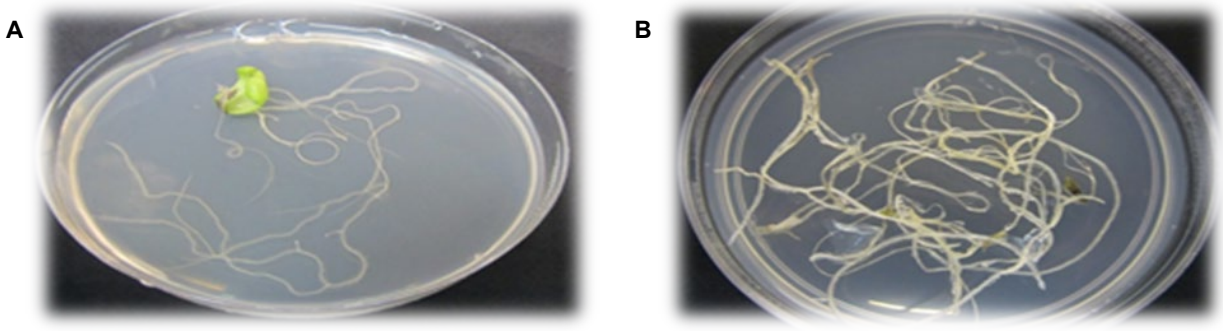

Figure 2: Hairy roots production on the leaves of oilseed rape (*B. napus* L.) plants seedlings inoculated with bacteria *A. rhizogenes* ATCC15834 through co-cultivation and its cultures. A: Hairy roots formed on leave incubation period 4 h with density (1.151 × 10^8 cell/mL); B: Culture of hairy roots developed from A.

Table 3: DNA concentration and purity isolated from genetically modified tissues of oilseed rape (*B. napus* L.) plants.

DNA source	Concentration ng μl^{-1}	Purity
Hairy root transformed by direct injection with 1.151 × 10^8 cell/mLof bacteria *A. rhizogenes* ATCC15834	318.7	1.8
Hairy root transformed by incubation period 4 h with bacteria *A. rhizogenes* ATCC15834 by co-cultivation	272.9	1.8
Seedlings roots (control)	287.6	1.7

the level of their internal auxins (Sharma *et al*., 2019). Transfer of T-DNA from Ri plasmids to the genetic material of the plant, their conjugation and their subsequent gene expression is responsible for the production of hairy roots from pieces of leaves at the injection sites of *A. rhizogenes* ATCC15834 (Keshavareddy *et al*., 2018).

Co-cultivation method

The results of incubating the leaves of oilseed rape with inoculum density of 1.151 × 10^8 cells/mL of *A. rhizogenes* ATCC15834 at different periods (2, 4 and 6 h) and the success of hairy root formation under all incubation periods for leaves and the superiority under 4 h incubation time with a ratio that reached 44.44% after 30 days of incubation is shown in Table 2. According to the data, co-cultivation was less efficient than the direct injection process.

The hairy roots are characteristic by their white color and their rapid growth (Figure 2A).

Production of bacteria-free hairy root cultures

The results of the transferred 0.5 g of hairy roots that formed on leaves as a result of inoculation with *A. rhizogenes* ATCC15834 via direct injection and co-cultivation methods on solid MS medium showed their ability to continue growing, superabundant, increase its branches and show negative geotropism in addition to gaining the ability of continual growth which leads to producing a typical culture (Figure 1C, 1D and Figure 2B), compared to the normal roots (control) that were placed

Figure 3: Chromosomal DNA isolated from hairy roots of oilseed rape (*B. napus* L.) transformed by bacteria *A. rhizogenes* ATCC15834. Lane 1: DNA isolated from seedlings roots (control); Lane 2: DNA isolated from transformed hairy root by direct injection with 1.151 × 10^8 cell/mL of bacteria *A. rhizogenes* ATCC15834; Lane 3: DNA isolated from transformed hairy root by incubation period 4 h with bacteria *A. rhizogenes* ATCC15834 by co-cultivation.

on the same medium but did not continue to grow, wilt and die later. The results showed that transferring the cultures of hairy roots for 3-4 transfers on MS solid medium containing 250 mg/L cefotaxime led to the elimination of the bacteria, as indicated by the absence of bacterial growth after three days of inoculation in YEB liquid medium with the mash of the hairy roots.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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DOI: http://dx.doi.org/10.21161/mjm.230241

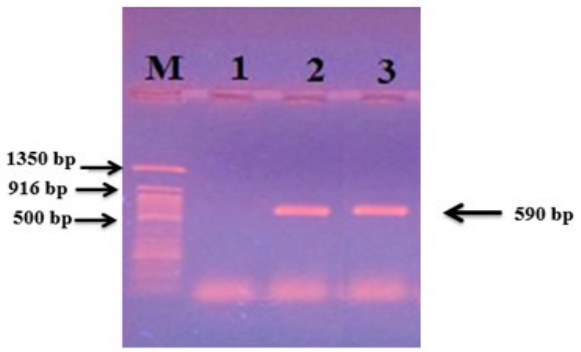

Figure 4: Electrophoresis of amplified DNA isolated from oilseed rape (*B. napus* L.) transformed tissues with bacteria *A. rhizogenes* ATCC15834 by direct injection and co-cultivation. M: DNA lambda; Lane 1: DNA isolated from seedlings root (control); Lane 2: DNA isolated from transformed hairy root by direct injection with 1.151×10^8 cell/mL of bacteria *A. rhizogenes* ATCC15834; Lane 3: DNA isolated from transformed hairy root by incubation period 4 h with bacteria *A. rhizogenes* ATCC15834 by co-cultivation.

Hairy roots proved to have more genetic stability than normal cells and did not require the addition of growth regulators in the medium in which they grew due to the length of their meristematic parts and the increased rates of their division (Meyer *et al*., 2000).

The elimination of bacteria is one of the very important processes necessary to prevent the survival of bacteria, induce the cultures of genetically modified tissues and stabilize their growth later. The efficiency of cefotaxime in removing bacteria from hairy root cultures is explained by its effect on the heterogeneity of the osmosis of bacterial cell membrane and in the pathways for building proteins, enzymes, the replication of nucleic acids and the division processes of bacterial cells that lead to their death (Venkatachalam *et al*., 2011).

Concentration and purity of the DNA isolated from different tissues

The data in Table 3 showed the superiority of DNA isolated from hairy root culture initiated by direct injection with *A. rhizogenes* ATCC15834 in its concentration that reached 318.7 ng/µL compared to the other types of DNA samples. The purity of all types of DNA is clearly shown from the sharp bands shown in Figure 3.

Preservation of the genome of transgenic oilseed rape tissues with *rol*C gene

The results of PCR confirmed the presence of one band with a size of 590 bp for DNA isolated samples of two types of hairy roots, similar to the molecular weight of the specific primer *rol*C used in this study (Figure 4) and the absence of such band in the untreated root samples (control). This result confirms the genetic transformation of these samples with T-DNA-*rol*C genes that are found on the Ri plasmids of *A. rhizogenes* ATCC15834 and their inclusion in plant genome.

The successful transfer of T-DNA genes from Ri plasmid into the plant genome led to an increase in cell divisions, which may explain the increase in DNA concentrations in genetically transformed samples (Park *et al*., 2010). Finally, our results confirmed by PCR, which gave a conclusive molecular improvement of the success of the genetic transformation and genetic expression of those genes in the oilseed rape cell genome (Balasubramanian *et al*., 2018; Lacroix and Citovsky, 2019; Sultan and Mohammed, 2020).

CONCLUSION

The current study succeeded in producing hairy roots from oilseed rape *B. napus* L. seedlings leave using direct injection and co-cultivation methods by *A. rhizogenes* ATCC15834.

ACKNOWLEDGEMENTS

The authors are very grateful to the University of Mosul/ College of Science and Al-Noor University College for the facilities they provided, which helped improve the quality of this work.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538