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GENETIC TRANSFORMATION OF *NIGELLA SATIVA* L. PLANTS WITH *AGROBACTERIUM RHIZOGENES 35S GUS* R1000 AND ESTIMATION OF THYMOQUINONE LEVEL IN TRANSFORMED HAIRY ROOTS CULTURES

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Abstract

Current study investigated the requirements to induce hairy roots on decapitated stems segments of Black cumin, *Nigella sativa* L. seedlings, *via Agrobacterium rhizogenes* R1000 contain 35S *GUS* genes. The direct injection of stems segment by bacterial inoculum showed it efficient and sustained for production the hairy roots within 20-25 days on inoculated and non-inoculated position with percent 33% at 4.2 hairy roots per segment. The hairy roots as single or clusters excised and placed on solidified MS medium supplemented with Cefotaxime at gradual concentrations 300, 200, 100 mg / L with subsequent transfers for elimination the bacteria and obtain its cultures of hairy roots. This phenotype of hairy roots were growing at fast, white in color and negatively geotropism. The staining of hairy roots with X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) showed it acquirement the dark blue color which gives the confirmation of genetic transformation of these roots. Furthermore, the hairy roots showed highest content of Thymoquinone (TQ) reached to 6.31mg/g comparing with seeds and normal seedlings 4.19, 1.27 mg/g respectively.

Key words: *Nigella sativa* L., *A. rhizogenes*, *β-Glucuronidase,* Hairy roots culture.

Introduction

The transgenic organisms having genetic material that acquire by molecular cloning techniques must contain a transgene in all cells, this means that the gene must be stably replication along with its genomic in every cell cycle (Fahimi *et al.,* 2016). There are many techniques of genetic transformation which develop and led to create genetically modified for different plants species which can tolerate to environmental stresses, increase productivity and quality.. (Alikina *et al.,* 2016; Souza *et al.,* 2017). *Agrobacterium rhizogenes* is one of the important tools for genetic transformation of plants, including transfer T-DNA genes and integration into plant cells genome, depend on the autonomous of Ri-plasmid (root-inducing) and product of hairy roots (Deng *et al.,* 2011; Al-Mallah and Mohammed, 2012). Reporter genes are useful especially for easy detection of transformation. Most commonly used reporter genes are *β-Glucuronidase* (*GUS*), *Chloramphenicol acetyl transferase* (*CAT*), *Nopaline synthase* (*NOS*), *Octopine synthase* (*OCS*) and *firefly luciferase* (*Lux*). The *β-Glucuronidase* (*GUS*) is an enzyme of bacterial coded by the *Escherichia coli* gene, *uidA* (also known as *gusA*). *GUS* gene has stable expression in the transformed leaves/ shoots/roots assayed histochemically (Ruhullah *et al.,* 2017). *Nigella sativa* L. plants be-long to *Ranunculacea* family, is an annual herb have pharmacological properties (Alireza *et al.,* 2017; Neamah, 2018) and considered as a good source of functional constitutes, has received increasing interest due to its healing effect against some diseases. Thymoquinone is the main bioactive constituent of an oil extract of *Nigella sativa* and show anti-inflammatory, anti-oxidant and anti-tumor activity (Woo *et al*., 2012; Khander and Eckl, 2014).

The present paper aimed to find the efficiency of 35S *GUS A. rhizogenes* R1000 to create transgenic hairy roots on Black cumin (*Nigella sativa* L.) plants. Also determination the Thymoquinone level in transformed hairy roots cultures.

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Table 1: Number, transformation frequency and average of initiated hairy roots from decapitated seedlings stems of *Nigella sativa* plants inoculated with *35S GUS A. rhizogenes* R1000

Age of bacteria (h) / density (cell/ml)	Number of Inoculated Segments	Transformation frequency %	Hairy root production %	Average of initiated hairy roots/segment	Transformation frequency %
DW (control)	25	0	0	0	0
48/215	100	39	33	4.2	33

For control, other sample inoculated with sterilize distill water.

Materials and Methods

Production of aseptic seedlings

Seeds of Black cumin, *Nigella sativa* L. provided from local market in Mosul /Iraq, surface sterilized by soaking in 3% sodium hypochlorite (NaOCl) for 5 min (Al-Ani, 2008). Then, placed on the surface of agar-solidified MS (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.8% agar with pH at 5.8. Specimens were maintained in culture room condition at 22±2°C in dark and when seedling grown, they transferred to the 16 hour light of florescent tube at density 41 μmol.m^{-2}.sec^{-1}.

Direct injection of Black cumin seedlings stems with *35S GUS* labeled *A. Rhizogenes R1000*

Fifteen days old Black cumin seedlings stems were excised and direct injected with inoculum of 35S *GUS*-labeled *A. rhizogenes* R1000 (48 hours age at density 215×10^8 cell /ml) using minute needle at 4 or 5 positions (AL-Mallah and Masyab, 2014). Inoculated seedlings were placed vertically in flask contain 25 ml of 1/2 MS medium. All flasks incubated at 25±2°C in the dark (Rajesh *et al.,* 2007).

GUS Staining of seedlings stems and hairy roots

GUS stain was prepared from 0.75ml of X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) at concentration of 20 mg ml^{-1}, in DMF (Dimethylformamide). 14.25 ml of X-Gluc buffer added to this to create 1.0 in 20 X-Gluc stain solution (Filipecki and Malepszy, 2006; Sambrook and Russel, 2011). Inoculated stems after 7 days were excised from the media, soaked in a flask with 2.0ml of X-Gluc stain. The flask was kept overnight at 37°C for initiation of stain, then replaced the stain by water. the same protocol was carried out with the hairy roots which initiated on stems.

Transgenic hairy root culture formation

A single root or a tuft of roots formed on the inoculated sites were removed and transferred to a 25mm diameter petri dishes containing ½ MS medium and to heal transformed hairy roots from bacteria, the roots were sub-cultured 4-5 times on MS media supplemented with progressive ascending of 100, 200, 300 mg/L of cefotaxime antibiotic with a period between each sub culture was15 day.

Table 2: Thymoquinone concentration in different extracts of *N. sativa* L.

Samples	Concentration (mg/g)
N. sativa seeds	4.19
Transgenic hairy roots	6.31
Normal seedlings roots	1.27

Determination of Thymoquinone concentration

5.0 grams of *N. sativa* seeds, transgenic hairy roots and normal seedlings roots extracted individually, by percolation at room temperature with methanol till exhaustion. The solvent was evaporated using Rotavapor. The residue was solved gradually with 50 mL methanol (Alam *et al*., 2013). Every one of above samples measurement in UV-Visible Spectrophotometric at 265 nm and the TQ concentration determined according to Beer-Lambert law.

$A= \varepsilon$ bc

A = the value of absorbance

ε = the constant of absorptivity

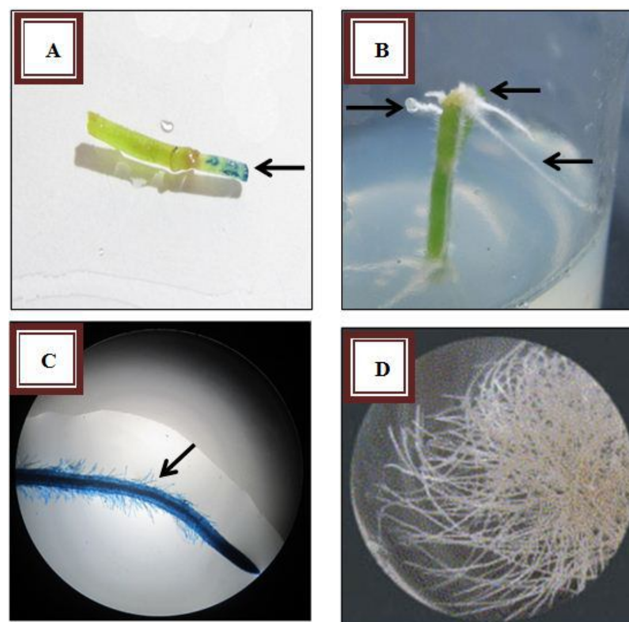

Fig. 1: Transformation hairy roots on Black cumin (*Nigella sativa* L.) seedling stem with *A. rhizogenes GUS* R1000 and formation of hairy root culture.

A. *GUS* stained of seedling stem segment after 7 days of inoculation. B. Hairy roots formation on the seedling stems (arrows) number and length of hairy roots after 15 days. C. *GUS* stained hairy root (arrows) under light microscope. D. Hairy roots culture grown on agar-solidified MS medium.

b = constant length of light beam passes through

c = solute concentration. (Behera *et al.,* 2012).

Results and Discussion

The results indicate efficiency of injection method by 48 h old bacterial inoculum at density 215×10^{8} cell /ml (Table 1) and sustained through the blue color existence in inoculated stem segments after staining with X-glue within 7 days from inoculation (Fig. 1A).

Hairy roots produced on the segment stems after 20-25 days (Fig. 1B), continued in growth and developed with percent 33%, at mean 4.2 hairy roots/segment.

The initiation of the hairy roots on the stems with the absence in the control samples belong to the transfer of T-DNA genes from the Ri-plasmids of *A. rhizogenes* to the genome of the plant cells and their integration with it and successes in gene expression (Rangslang *et al*., 2019). Also, staining with X-Gluc, where showed clear blue color, light microscope exanimation of the blue-colored transgenic hairy roots support the expression of 35S *GUS* gene (Fig. 1C). The acquisition of transgenic tissues with the blue color belong to the expression of the *GUS* gene in *A. rhizogenes* R1000 and the promoter 35s CaMV promoter as responsible for changing the color of the colorless X-Gluc dye solution to blue when incubating the stem and hairy roots, which was induced by the *GUS-A. rhizogenes* R1000, in this dye (Gallagher, 1992).

The data showed, removing single root or cluster from the stem segments and cultured on the of solid MSO medium, led to formation of dense mass and developed to the good cultures of those roots. While the seedlings roots (control) failed to continue to grow on the same medium. The hairy roots in this cultures characterized by fast growing, white, dens of root hairs and negatively geotropism growth (Fig. 1D). The continued growth of well cultures without the need for any addition of growth stimulation is additional evidence for the genetic transformation of these tissues (Al-Mallah and Mohammad, 2012). This technology has been important due to it using in various applications (Deng *et al.,* 2011).

The values of Thymoquinone concentrations estimated in this study clearly superiority of its in transgenic hairy roots samples reached to 6.31 mg/g of tissues (Table 2) comparing with seeds and normal seedlings roots with 4.19, 1.27 mg/g respectively.

The hairy roots are a good source for accumulation of secondary metabolites due to the role of *rol* gene which present in T-DNA of Ri plasmid, when integrated to plant cell genome (Bulgakov, 2008; Pala *et al*., 2016). Such as *A. dubia* when enhanced over production of artemisinin (Kiani *et al.* 2012).

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