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

Amjad Abdul-Hadi Mohammed¹ and Hikmat Mustafa Masyab²*

¹Department of Biology, College of Science, University of Mosul, Mosul, Iraq.
²Department of Biology, Faculty of Science and Health, Koya University, Kurdistan Region, Iraq.

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 Ranunculaceae family, is an annual herb have pharmacological properties (Alirez 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.

*Author for correspondence : E-mail: hikmat.mustafa@koyauniversity.org
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 was 15 day.

**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.

\[
A = \varepsilon \cdot bc
\]

\( A \) = the value of absorbance

\( \varepsilon \) = the constant of absorptivity

**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 \( \mu \)mol.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 ½ 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-\( \beta \)-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

---

**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

<table>
<thead>
<tr>
<th>Age of bacteria (h) / density (cell/ml)</th>
<th>Number of Inoculated Segments</th>
<th>Transformation frequency %</th>
<th>Hairy root production %</th>
<th>Average of initiated hairy roots/segment</th>
<th>Transformation frequency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>DW (control)</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>48/215</td>
<td>100</td>
<td>39</td>
<td>33</td>
<td>42</td>
<td>33</td>
</tr>
</tbody>
</table>

For control, other sample inoculated with sterile distill water.

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. sativa</em> seeds</td>
<td>4.19</td>
</tr>
<tr>
<td>Transgenic hairy roots</td>
<td>6.31</td>
</tr>
<tr>
<td>Normal seedlings roots</td>
<td>1.27</td>
</tr>
</tbody>
</table>

---

**Fig. 1:** Transformation hairy roots on Black cumin (*Nigella sativa* L.) 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 215x10^6 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 examination 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).

Acknowledgment
The authors are very grateful to the University of
Mosul, College of Science for their provided facilities,
which helped to improve the quality of this work.

References
Alam, P., H. Yusufoglu and A. Alam (2013). HPTLC
densitometric method for analysis of thymoquinone in
Nigella sativa extracts and marketed formulations. Asian
Alireza, T., M.R. Bibi, A. Ali and H. Hossein (2017). Black Seed
(Nigella Sativa) and its Constituent Thymoquinone as
an Antidote or a Protective Agent Against Natural or
Chemical Toxicities. Iranian j. pharmaceutical res.,
(IJPR) 16(Suppl): 2-23.
Al-Ani, N.K. (2008). Thymol production from callus culture of
Nigella sativa L. Plant Tissue Cult. and Biotech., 18(2):
181-185.
Alikina, O., M. Chernobrovkina, S. Dolgov and D.
Miroshnichenko (2016). Tissue culture efficiency of wheat
species with different genomic formulas. Crop Breeding
and GFP reporter genes in transgenic hairy roots of tomato
and potato plants via Agrobacterium rhizogenes mediated
AL-Mallah, M.K. and A.A. Mohammed (2012). Transfer of Ri T-
DNA genes of Agrobacterium rhizogenes R1601 via direct
microinjection and Co-cultivation to carrot, Daucus carota
L. tissue and production of transformed hairy root cultures.
Iraqi J. Biotech., 11: 227-239.
Behera, S., S. Ghanty, F. Ahmad, S. Santra and S. Banerjee
(2012). UV-Visible spectrophotometric method
development and validation of assay of paracetamol tablet
Bulgakov, V.P. (2008). Functions of rol genes in plant
composite plants in Medicago truncatula used for
Transgenic Animal Technology: Technique and Its
Application to Improve Animal Productivity. Adva. Life
of plant transformation: a molecular insight. J. Appl. Genet.,
a Reporter of Gene Expression. Academic Press Inc. pp215,


