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GUS-gene as a Visual Marker for *Gluconacetobacter diazotrophicus* Co-cultivated with Carrot Plantlets

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ABSTRACT

This investigation assessed the interaction between carrot, *Daucus carota* l., axenic seedlings and N_2-fixing bacteria *Glucanacetobacter Diazotrophicus* labeled with *GUS*-reporter gene. The results indicate the improvement of growth of the inoculated seedlings without the formation of nodule fixing atmospheric nitrogen. Transformed tissue stained by X-Gluc solution acquired the blue color and visualized by naked eyes. This is the most important signs which represent a strong marker pointed out the successful expression of *GUS* (β-glucuronidase) gene. This gene considered one of the reporter genes used for the first time in determining the efficiency of gene expression between carrot and this type of N_2 fixing bacteria.

Keywords: *GUS*-gene, *Gluconacetobacter diazotrophicus, Carrot.*

1. INTRODUCTION

Gluconacetobacter diazotrophicus ,labeled with *GUS* –gene ,belong to Acetobacteraceas family , Gram-negative,rod of 0.7-0.9X1.0-2.0µm in dimension .(Gills et al 1989).Aerobic,Fixing N_2,free living (Swings 1992).This bacteria conlonized in host plant tissue and secreted half of the fixed N_2 as available energy source for plant to improve its growth .(Cojho *et.al* ,1993).Since ,this bacterial species improving plant growth because of its potentiality in fixing atmospheric N_2 without root nodule and absence of host specificity. In this relation ,therefore many investigators isolated this bacteria from many plants such as *Coffea arabica* L.,*Musa spp*, *Ananas comosus* ,*Mangifera indica* L. grown in field. This type of bacteria not available in soil, probably present in roots and stems and other plant organs .It observered in leatf of sugarbeet (Reis *et,al* 1994),Xylem tissue (James *et,al* 2001).and in guard cells (Al-Nema *et,al* 2014) of the same plant .*GUS*-gene was first isolated from *Escherichia coli* (Jefferson *et,al* 1986). It was used as genetic marker in plant Co-cultivated with bacteria (Wilson *et,al* 1992). A study success in observation this *GUS*-labeled *G.dizotrophicus* isolated from sugarcane and some field crops such as *Zea mays* L., *Arabidopsis thialaua* (Cocking *et,al* 2006). And from transformed hairy roots by using X-Gluc stain that acquired the blue color .This is a marker for the *GUS*-gene transfer with other bacterial genes to genome of plants inoculated with *Agrobacterium rhizogenes* (park *et,al* 2010). This study aimed to determine the carrot –*Gluconacetobacter diazotrophicus* interaction by *GUS*- gene as visual marker.

2. METHODOLOGY

Plant Materials

Seeds of local variety (orange color) of carrot, *Daucas carota* L., were surface sterilized by soaking in solution of the commercial bleach ("Fas" Babylon Company for detergent, Baghdad, Iraq) for 5 minutes then they thoroughly washed by sterile water (Al-Mallah and Mohammed 2012). Sterilized seeds were sown in 250 ml capacity flasks on the surface of 50 ml of hormone-free agar-solidified MS (Murashige and Skoog 1962) medium. Specimens were kept in culture room at condition of 25±2 C° ,16 h. light \ 8h. dark, 1500 lux light intensity.

Gluconacetobacter diazotrophicus* **and culture conditions.**

G.diazotrophicus was provided from Prof. E.C.Cocking, Faculty of Biological Sciences, University of Nottingham UK. This organism was labeled by *GUS* gene fused with its genome. Bacterial suspension was prepared in AT-*GUS* liquid medium (0.9% w\v agar, yeast extract (2.7 g/l), Glucose (2.7 g/l), mannitol (1.8g/l), MES buffer (4.4g/l), K_2HPO_4 (0.65g/l), pH 6.3) containing 4.5 mg as required. Expression of the β-glucurondiase (*GUS* A) gene was tested by plating on AT-*GUS* medium containing X-Glue (5-bromo-4-chloro-3-indolyl-beta-D-gluconic acid liquid medium and a dilution of O.D. equal to 1.2 was produced (Stone *et.al.* 2001).

Inoculation of plantlets with *G.diazotrophicus*

The dilution of 10^9 cell/ml of *GUS*-labeled *G.diazotrophicus* suspension was used in inoculation of 2-weeks old sterile carrot seedlings. For each five seedlings grown on agar-solidified MS medium 1.0 ml inoculum was added, and to other five seedlings 1.0 ml of distilled water was poured to represent the control (Al-Nema *et.al* 2014). Specimens were kept in culture room at conditions mentioned previously.

Staining with X-Gluc.

Five days after carrot seedlings were inoculated with *G.diazatropicus*. Other five unenoculated seedlings (control) were removed carefully from culture. The inactat inoculated seedlings were placed in 15 ml capacity glass jars containing 5 ml of freshly prepared X-Gluc solution (Fuentes-Ramires *et.al.* 1999). Un-inoculated seedlings were treated by identical manner. Both unlocked sample jars were placed in dessicator under vaccum for 30 min. Then samples were removed and kept in dark at 37C° for 24 h.(Cocking *et.al.* 2006,Al-Nema *et.al* 2014).

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Light Microscopy

Anatomical temporary preparations were made in leaves stained with blue color and control specimens. They were examined under Nikon light microscopy (Cocking *et.al.* 2006). Intact blue colored leaves and seedlings were photographed as well.

Re-isolation of bacteria from roots

Roots of inoculated and uninoculated carrot seedlings were excised and surface sterilized for 5.0 min. in 3%(v:v) "Fas" bleach. Then rinsed thoroughly with sterile water, and macerated in 2.0 ml sterile water (Al-Mallah *et.al* 1987) diluted samples were taken from macerated supernant and placed on AT-*GUS* medium with or without addition of streptomycin (45 mg/L). Also inoculated and un-inoculated plants were treated in similar way but without surface sterilization with "Fas" bleach and without rinsing. They placed directly on to the same AT-*GUS* medium to assess the efficiency of surface colonization,(Cocking *et.al.* 2006).

3. RESULTS

Production of axenic seedlings

Healthy carrot, *Daucus carota* L. seedlings were obtained from sterilized seeds grown on agar-solidified MS medium. They were used at 2 weeks of fully expanded leaves this study with.

Inoculation of seedlings with *GUS*-labeled *G.diazotrophicus*.

Observations indicate that the inoculum dilution 10^9 cell/ml of this bacterium was effective for infection. The data in table (1) referred to the successful infection of carrot seedlings.

Table (1): Inoculation of carrot, *Daucus carota*, seedlings by *Gluconacetobacter diazotrophicus* grown on agar solidified MS medium.

Treatments	No. of seedlings Inoculated/Infected	Infection (%)
Inoculated	25/20	80
Uninoculated (Control)	10/0	0

Moreover, it was noted clearly that growth of infected seedlings were improved. This is including shoots height and they had typical green color. While the uninoculated (control) seedlings posses less number of shoots.

Detection of *GUS*-gene by X-Gluc.

The results indicate that the color of inoculated seedlings soaked for 24h. in X-Gluc stain solution changed from green to blue. Whereas the color of the un-inoculated seedlings not change when immersed in this solution (Table 2).

Table (2): Staining of inoculated and un-inoculated carrot, *Daucus carota,* seedlings and leaves with X-Gluc

Samples	No. of stained samples	No. of samples colored blue
Inoculated seedlings	25	20
Un-inoculated seedlings	10	0.0
Individual inoculated leaf	25	25
Individual uninoculated leaf	10	0.0

The results showed that the colorless of X-Gluc solution change to blue. Also the inoculated carrot seedlings appeared in blue color as well (Fig.1.A). The un-inoculated seedlings kept the green color (Fig.1.B) when incubated separately in X-Gluc stain solution. Again, the same occurred with leaves excised from inoculated (Fig.1.C) and un-inoculated seedlings (Fig.1.D).

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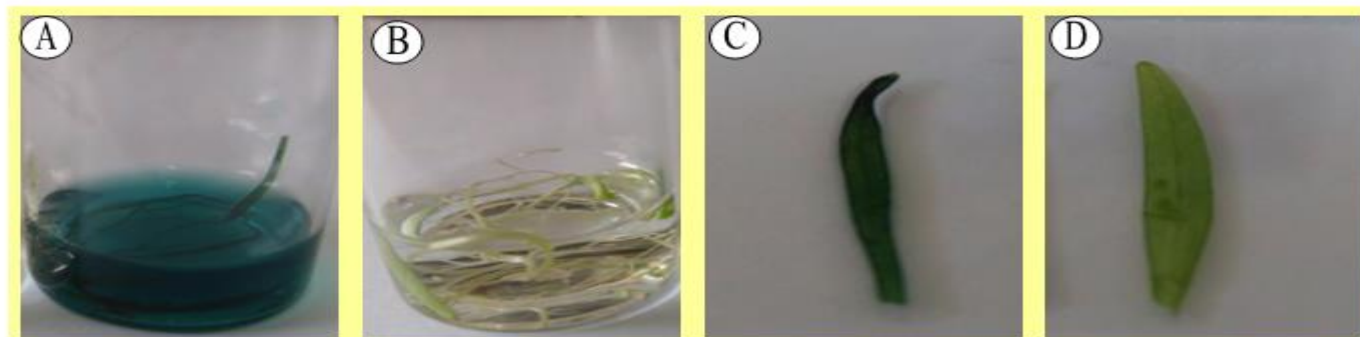

Fig. 1. Assessment of *GUS* gene in inoculated and un-inoculated carrot *Daucus carota* , seedlings and detached leaves with *GUS*-labeled *Gluconacetobacter diazotrophicus* treated Gluc solution]

A: Inoculated blue color seedlings. **B:** Un-inoculated seedlings (control) of green color.
C: Detached leaf acquired blue color. **D:** Detached leaf kept its green color (control).

Light microscopic examinations of infected samples proved the presence of *G.diazotrophicus* bacteria in leaf tissues (Fig.2.A), and stems tissues (Fig.2.B), of the blue color samples.

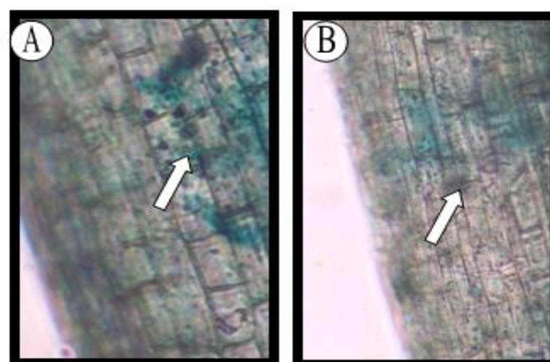

Fig. 2. Light microscopic photographs of root and stem tissues of *Daucus carota* inoculated with *Gluconacetobacter diazotrophicus*.
A:Presence of bacterium (arrowed) in cells of infected roots.
B:Prevaleance of bacterium (arrowed) in parenchyma cells of stems.

4. DISCUSSION

The successful interaction between *Gluconacetobater diazotrophicus*, fused with *GUS*-gene, and carrot seedlings refer to the arrival and co-exist of this bacterium in root cell walls (Lery *et.al* 2008).This may due to the formation of certain exopoly-saccharide by bacteria, which protect bacteria and enables it to fix N_2 (Dong *et.al.* 2002). Such as in corn, *Zea mays* and *Arabidopsis thialana* (Cocking *et.al.* 2006). Certainly, this may due to the expression of *GUS*-gene fused in *G. diazotrophicus* carried in *nif* H promoter-*GUS* A. The latter responsible for the color change of the colorless X-Gluc solution to blue color when inoculated carrot seedlings incubated in this stain. This may attributed to the formation of CLBr-indigo dichloro-dibromoiodigo that oxidized X-Gluc by β-glucuroridase (Caissard et al 1992).These conditions indicated that determinant factors of carrot-bacterium interaction were suitable for gene expression controlled nitrogenase enzyme (dos Santos *et.al.* 2010). Data obtained from microscopic examination sustained carrot-*G.diazotrophicus* interaction and emphasized that the bacterial invasion occur through the thin walls of the meristematic cells. (Al-Nema *et.al* 2014). In conclusion ,this study can be regard as an additional novel step toward establishing new relation between *G.diazotrophicus* and plants. Moreover, it indicates the bacterial efforts to survive, establish an interactive communication with plants and then infect them.

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REFERENCES

[1]. Al-Nema, Q. S.; Al-Mallah, M. K. and Cocking, E. C. (2014).An investigation of the interaction of *GUS*-labelled *Gluconacetobacter diazotrophicus* with sugarbeet (*Beta vulgaris* L.) seedlings. Int. J. Plant Biol. Res. (Submitted).

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[2]. Caissard, J. C.; Rembur, J. and Chriqui, D. (1992). Electron microcopy and X-ray microanalysis as tools for fine localization of β-glucuronidase activity in transgenic plants harbouring the *GUS* reporter gene. Protoplasma, 170: 68-76.

[3]. Cocking, E. C.; Stone, P. J. and Davey, M. R. (2006). Intracellular colonization of roots of *Arabidopsis* and crop plants by *Gluconacetobacter diazotrophicus*. *In Vitro* Cell. Dev. Biol. Plant, 42: 74-82.

[1]. Cojho, E. H.; Reis, M. V.; Schenberg, A. C. G. and Döbereiner, J. (1993). Interactions of *Acetobacter diazotrophicus* with an amylolytic yeast in nitrogen-free batch culture. Fed. Eur. Microbiol. Soc. Microbiol. Lett., 106: 341-346.

[2]. Dong, Z.; Zelmer, C. D.; Canny, M. J.; McCully, M. E.; Luit, B.; Pan, B.; Faustino, R. S.; Pierce, G. N. and Vessey, J. K. (2002). Evidence for production of nitrogen from O_2 by colony structure in the aerobic diazotroph *Gluconacetobacter diazotrophicus*. Microbiol., 148: 2293-2298.

[3]. Fuentez-Ramirez, L. E.; Caballero-Mellado, J.;Sepulveda, J. and Martinez-Romero, E. (1999). Colonization of sugarcane by *Acetobacter dazotrophicus*is inhibited by high N-fertilization. Fed. Eur. Microbiol. Soc. Microbiol. Eco. 29: 117-128. (C. F. Cocking *et al*., 2006).

[4]. Gillis, M.; Kersters, K.; Hoste, B.; Janssens, D.; Kroppenstedt, R. M.; Stephan, M. P.; Teixeira, K. R.; Döbereiner, J. and De Ley, J. (1989). *Acetobacter diazotrophicus*sp. nov., a nitrogen-fixing acetic acid bacterium associated with sugarcane. Int. J. Syst. Bacteriol., 39: 361-364.

[5]. James, E. K.; Olivares, F. L.; de Oliveira, A. L. M.; Dos Reis, F. B.; Da Silva, L. G.; and Reis, V. M. (2001). Further observations on the interaction between sugar cane and *Gluconacetobacter diazotrophicus* under laboratory and greenhouse conditions. J. Exp. Bot., 52: 747-760.

[6]. Jefferson, R. A.; Burgess, S. M. and Hirsch, D. (1986). β-Glucuronidase from *Escherichia coli* as a gene fusion marker. Proc. Natl. Acad. Sci., 83: 8447-8451.

[7]. Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant., 15: 473-497.

[8]. Reis, V. M.; Olivares, F. L. and Döbereiner, J. (1994). Improved methodology for isolation of *Acetobacter diazotrophicus* and confirmation of its endophytic habitat. World J. Microbiol. Biotechnol., 10: 401-405.

[9]. Stone, P. J.; O'Callaghan, K. J.; Davey, M. R. and Cocking, E. C. (2001). *Azorhizobium caulinodans* ORS571 colonizes the xylem of *Arabidopsis thialana*. Mol. Plant Microbe. Interact., 14: 93-97.

[10]. Swings, J. (1992). The genera *Acetobacter* and *Gluconobacter*. In: Balows, A.; Truper, H. G.; Dworkin, M.; Harder, W. and Schleifer, K., The Prokaryotes: A Handbook on the Biology of Bacteria. Vol. 3. Springer, New York. U. S. A. pp. 2268-2286.

[11]. Wilson, K. G.; Jefferson, R. A. and Hughes, S. G. (1992). The *Escherichia coli GUS* operon: Induction and Expression of the *GUS* Operon in *E. coli* and the Occurrence and Use of *GUS* in Other Bacteria. In: Gallagher, S. R., *GUS* Protocols: Using the *GUS* Gene As A Reporter of Gene Expression. Academic Press, INC., USA.

[12]. Caissard, J.C.; Rembur, J. and Chriqui, D. (1992). Electron microscopy and X-ray microanalysis as tools for fine localization of β-glucurnidase activity in transgenic plants harboring the *Gus* reporter gene. Protoplasma 170:68-76.

[13]. Al-Mallah, M.K.; Davey, M.R. and Cocking, E.C. (1987). Enzymatic treatment of clover root hairs removes a barrier to Rhizobium-host specificity. Bio / Techn. 5:1319-1322.

[14]. Al-Mallah, M.K. and Mohammed A.A. (2012). Transfer of T-DNA genes of Ri-plasmids in *Agrobacterium rhizogenes* R1601 through direct injection and Co-cultivation to carrot tissue and formation of genetically transformed hairy roots. Iraqi J. of Biotech. 11:227-239.

[15]. Park, N.; Park, J.H.; Lee, C.Y.; Lee, S.Y. and Park, S.U. (2010). *Agrobacterium rhizogenes*-mediated transformation of β-glucuronidase reporter gene in hairy roots of *Angelica gigus* Nakai. Plant Omics J. 3:115-120.

[16]. Dos-Santos, M.F.; dePadua, V.L.; Nogueira, E.M.; Hemerly, A.S. and Domont, G.R. (2010). Proteome of *Gluconacetobacter diazotrophicus* co-cultivated with sugarcane plantlets. J. Proteom. 73:917-931.

[17]. Al-Mallah, M.K. and Masyab, H.H. (2014) . Expression of *GUS* reporter genes in transgenic hairy roots of tomato and potato plants via *Agrobacterium rhizogenes*-mediated transformation. Aust. J. Appl. Sci. 8:234-239.

[18]. Lery, L.M.S.; Von Kruger, W.M.A.; Viana, F.C.; Teixeira K.R.S. and Bisch P.M. (2008). A comparative proteomic analysis of *Gluconacetobacter diazotrophicus* PAL5 at exponential and stationary phases of cultures in the presence of high and low levels of inorganic nitrogen compound. Biochimica et Biophysica Acta 1784 1578-1589.

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