Glucuronidase Gene: A Strong Evidence of a Novel Interaction of Glucuronidase-labeled Gluconacetobacter diazotrophicus with Spinach, Spinacia oleracea L. Seedlings

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Abstract—Gluconacetobacter diazotrophicus lives inside plant tissue cells in the form of colonies and excretes about half of the fixed nitrogen, which offers potential power that improves plant growth. The aim of this study is to find the interaction of glucuronidase (GUS)-labeled G. diazotrophicus with spinach seedlings and the detection of GUS genes using X-gluc dye (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid). The GUS protocol is used to detect GUS-labeled G. diazotrophicus in spinach seedling tissues by chemical detection using X-gluc dye. The results show that the spinach seedlings are successfully infected with GUS-labeled G. diazotrophicus, with the survival of the seedlings throughout their growth period and an improvement in the growth of pollinated seedlings. The outcomes of the microscopic inspection of the root slices reveal the presence of bacterial cells at the root tips and their concentration in the area of the cell walls of the peripheral cells. Furthermore, the findings of microscopic examinations of longitudinal sections for cotyledons show the presence of a number of bacteria within epidermal cell walls. This indicates that the determinants of the interaction between these bacteria and spinach seedlings are suitable for the expression of the gene responsible for the formation of the nitrogenase enzyme.

Index Terms — Glucuronidase protocol, Gluconacetobacter diazotrophicus, Interaction, Nitrogen fixation, Spinach.

I. INTRODUCTION
Spinach (Spinacia oleracea L.) is one of the leafy green vegetables rich in nutrients; it stores great quantities of carotenoids, Vitamin C, folate, Vitamin K, calcium, and iron (Furness, et al., 2013). Gluconacetobacter diazotrophicus bacteria were isolated for the first time from the roots and stems of sugarcane plants, Saccharum officinarum L., and it was previously called Acetobacter diazotrophicus. The endophytic, non-rhizobial bacterium has been similarly separated from other monocot plants (Eskin, Vessey, and Tian, 2014). The 15N2 tests demonstrated the ability of this bacteria to fix atmospheric nitrogen in sugarcane plants (Boddey, et al., 2003), thus acting as a biostimulant (Grillo-Puertas, et al., 2018). The use of reporter genes has become widespread nowadays with plant or animal tissues and with microorganisms as a quick and direct way to observe the expected results, and the most important genes are GUS (an enzyme from the bacterium Escherichia coli is a competing reporter gene that uses a histochemical technique to analyze promoter activity of an induced gene), NPT II, GFP, CAT, and LUC (Miki, 2008). One of the studies followed the GUS gene in genetically transformed hairy root cultures emerging from tomato and potato plants using the X-gluc dye and stained it with a blue color. This is evidence of the transmission of this gene with the genes of the Agrobacterium rhizogenes bacterial vector to the genome of plants inoculated with these bacteria. Then, the hairy roots emerge from them (Al-Mallah and Masyeb, 2014). This investigation aimed to find the interaction of GUS-labeled G. diazotrophicus with spinach seedlings and the detection of GUS genes using X-gluc dye.

II. MATERIALS AND METHODS
A. Seeds Growth and Plant Cultivation
Seeds of S. oleracea were exterior uncontaminated by soaking in 70% ethanol solution for 2 min and properly diluting “Domestos” bleach with 5% of sodium hypochlorite (Lever Fabergé; Kingston upon Thames, UK) 2:4, v:v for
30 min. Later, it was rinsed in sterile distal water (Shojaei, et al., 2010). Overlay disinfected seeds were grown in autoclaved vermiculite and antiseptic water for up to 5 days/28°C, 16 h photoperiod. After 5 days, seedlings (root approximately 1.0 cm in length) were transferred to sterile MSO (Murashige and Skoog, 1962), 3% sucrose, and 0.8% agar in small jars (one seedling per jar). They were kept in a growth room at 25°C with light for 4 days.

B. Preparation of G. diazotrophicus inoculum

Nine dilutions of GUS-labeled G. diazotrophicus were provided by Azotic Technologies, Ltd., Nottingham, United Kingdom; suspensions were prepared; and the optical density (O.D 600) was fixed at 1.2. G. diazotrophicus bacterium type 5541, UAP5541/pRGS561 expressing the GUS was cultured on AT-GUS medium (Sebring, et al., 2022).

C. Inoculation Procedures

The last dilution (10⁸) was used to inoculate 25 seedlings whose root system was in MSO medium by adding 1.0 ml of suspension to it. Twenty-five other seedlings were vaccinated by adding 1.0 ml of sterile distilled water only (comparison). Inoculated samples were kept in the development room under the same previous conditions (Rasheed, 2014; Ruhullah, et al., 2017).

D. Histochemical Staining using X-GLUC

Three of the bacteria-inoculated seedlings and three of the control seedlings, after 4 days of inoculation, were removed from the medium and their root aggregates were carefully removed from the suspended agar. Whole inoculated seedlings were placed in sterile 10 ml vials, including 5.0 ml fresh X-gluc dye mixture (Kong, et al., 2023). Similarly, the comparison seedlings were transferred to another tube containing the same volume of the same dye solution as well. The bottles were placed inside a desiccator container with the lids left open and connected to a vacuum pump for 30 min. Formerly, the sections were taken and saved in the darkness at 37°C for 24 h (Rasheed, 2014; Mohammed and Masyab, 2020).

E. Direct Microscopic Observation

The steps for preparing all histological sections of inoculated plant samples were carried out according to Cocking, Stone and Davey (2006). At each sampling time, roots and cotyledon leaves were excised, and histochemical staining was done through X-gluc and seen by light field microscopy. To observe, slices of roots and cotyledon leaves, samples displaying blue color, were restored in glutaraldehyde (2%) with dissolved sodium phosphate (0.1 M) buffer reaching 24 h in the refrigerator, dried by ethanol, and set in white medium-grade acrylic resin (Agar Aids, UK). Later, sections with 1.4 μm thickness stained with safranin (0.01%), and observed under 10X and 100X objective lenses. Experiments were repeated (histochemical staining and observation) after 12 days of inoculation (Ruhullah, et al., 2017).

III. Results

The results recognized that intracellular occupation for the tips of the root appears after injection of spinach plants through G. diazotrophicus. It was also found for all samples examined that the most extensive intracellular colonization was in shoots and roots cultivated in a semisolid MS medium. Yet young seedlings grown on MS agar medium with 10⁹ CFU were inoculated, the quantity typically runs down for inoculations of plants by G. diazotrophicus strain UAP5541.

Data showed that the color of the X-gluc dye solution after 24 h was changed from colorless to blue for inoculated seedlings and remained colorless for un-inoculated seedlings. Furthermore, the leaves of inoculated seedlings were dyed blue compared with the un-inoculated seedling leaves.

Microscopic assessments for root tips verified the existence of bacterial cells within the cell walls of these roots 4 days after inoculation through GUSA–G. diazotrophicus (Fig. 1a), whereas the absence of bacteria was observed in the root tips of control samples (Fig. 1b). Pieces of roots that are embedded in resin 12 days after inoculation

![Fig. 1. Illumination micrographs of roots of Spinacia oleracea L. inoculated with Gluconacetobacter diazotrophicus 4 days and 12 days after inoculation and un-inoculated control. (a) Root section inoculated through GUSA–G. diazotrophicus viewing bacteria inside the cells and within the walls (arrows) 4 days after inoculation. (b) Longitudinal section of the stained un-inoculated root tip (control). (c) Root section inoculated through GUSA–G. diazotrophicus screening bacteria inside the cells (arrows) 12 days after inoculation. (d and e) Section of root inoculated through GUSA–G. diazotrophicus, displaying widespread intracellular occupation and the existence of micro-colonies (arrows). (f) Root apex peripheral cells stained section of un-inoculated (control). Scale bars ¼ 10 mm (a-f).]
through GUSA–G. diazotrophicus presented dim blue-dyed G. diazotrophicus inside the cells and similarly in the cell walls (Fig. 1c). Bacterial invasion of the elongation zone and their spread were heavy (Fig. 1d and e). The absence of dim blue-dyed G. diazotrophicus was noted within stained slices of un-inoculated root tips (control) (Fig. 1f).

Moreover, the results demonstrated the presence of bacterial cells within cellular walls for cotyledon leaf epidermal cells (Fig. 2a); in some sections, bacterial cells were closely associated with vesicles (Fig. 2b). Data from microscopic observations detected leaf cells’ intracellular occupation with blue-dyed G. diazotrophicus clearly (Fig. 2c) and also viewed numerous bacterial cells within the guard cells (Fig. 2d). Again, the absence of dim blue dyed G. diazotrophicus in divisions of un-inoculated dyed leaves (control) (Fig. 2e and f).

**IV. DISCUSSION**

The success of the interaction of spinach seedlings with GUS-labeled G. diazotrophicus bacteria and the improvement in its growth reflects the access of this bacterium to the cell walls of the roots and its concentration among these cells. Their concentration between these cells was known for its effective role in resisting the diffusion of oxygen to enable it to fix nitrogen in conditions of high concentrations of oxygen, thus preserving the effectiveness of the nitrogenase enzyme from the flow of oxygen (Dong, et al., 2002). The mutual signals in the response between the plant and then the G. diazotrophicus bacteria, which enabled bacteria to penetrate the plant cell walls and then settle inside cells, may be attributed to the fact that the inoculation and growth conditions in the MS medium containing sucrose were suitable for both bacteria and plants (Rodriguez, et al., 2019). It is likely that the contributing reason for this point is that G. diazotrophicus creates great quantities of indole acetic acid, and plant development is usually inhibited through extreme intensities of auxin hormone (Rocafull, et al., 2016). However, small quantities of IAA are known to function such as a common signifying iota in plant and bacteria relations, with microbial-liberated IAA stimulating a reaction differing from useful to harmful in plant cells. This is determined by the endogenous level of IAA in the root cells (Duca, et al., 2014). Subsequent cell wall access through G. diazotrophicus, intracellular bacteria occupation in vesicles might produce as sucrose caused the endocytosis (Rodriguez, et al., 2019). Exopolysaccharides manufactured through G. diazotrophicus are in charge of mucoid bacteria development (Lambrecht, et al., 2000). G. diazotrophicus culture is able to utilize a mucoid medium as an operative challenge to the diffusion of oxygen, allowing bacteria cells to do nitrogen fixation in a great range of oxygen levels, thus keeping nitrogenase action from extreme oxygen change (Dong, et al., 2002). In general, this is due to GUS gene expression carried in G. diazotrophicus which is loaded with nifH promoter and GUSA promoter, which is in charge of changing the color of the X-gluc dye from colorless to blue when incubating the inoculated spinach seedling (Masyab, 2018). Herein, dye explains the color change to the formation of dichloro-dibromoindigo (ClBr-indigo) compound as a result of the oxidation of X-gluc dye by β-glucuronidase (GUS) enzyme (Xiong, et al., 2011). This proves that the interaction determinants between these bacteria and spinach seedlings were suitable for the expression of the gene responsible for forming nitrogenase enzyme (Varghese, et al., 2019). Whereas, the similar G. diazotrophicus nifH–GUSA form existed within dead wood vessels of sugarcane-negative GUS expression was identified (Eskin, Vessey et al., 2019). Prospect acts through mutants influenced exopolysaccharide production (Sevilla and Kennedy, 2000). This might aid to verify if exopolysaccharides are in charge of the development of G. diazotrophicus; moreover, nifH gene expression is associated with exopolysaccharide production (Dietz, 2022). The lengthy pleomorphic cells of intracellular G. diazotrophicus are a feature of G. diazotrophicus development at great concentrations of nitrogen (NH\textsubscript{4}NO\textsubscript{3}, NH\textsubscript{4}Cl, KNO\textsubscript{3}, and Urea) representing in MS media as a source for plant development (Muthukumarasamy, Revathi and Loganathan, 2002). The G. diazotrophicus has extreme tolerance to acid, growth, and nitrogen fixation much at the pH (3.0) and less than that (Grillo-Puertas, et al., 2018).

**Fig. 2.** Illumination micrographs of cotyledon leaves for Spinacia oleracea L. inoculated with Gluconacetobacter diazotrophicus 4 days and 12 days after inoculation and un-inoculated control. (a) Inoculated leaf section through GUSA–G. diazotrophicus viewing bacteria inside the cells beside the cell walls (arrows). (b) Leaf section at 4 days after inoculation through GUSA–G. diazotrophicus, displaying stretched pleomorphic bacterial cells in addition to massive intracellular occupation for leaves cells through bacteria strictly coupled with vesicles (arrows). (c) Intracellular colonization in leaf displaying blue-stained G. diazotrophicus within cells (arrows). (d) Bacterial cells were invading guard cells (arrows). (e and f) Cotyledon un-inoculated section stained (control). Scale bars ¼ 10 mm (a-f).
Furthermore, ammonia (NH₄) affects incomplete inhibition for nitrogenase lone (Medeiros, Polidoro, and Reis, 2006). The collaboration between non-rhizobial G. diazotrophicus bacteria together with the apex of a plant root has seen firm resemblances to that taking place among angiosperm genera and nitrogen-fixing cyanobacteria such as Nostoc sp. (Chiu, et al., 2005). These cyanobacteria enter the meristematic cells through their thin walls at the stem by liquefying the wall of cells and then are immersed in vesicles within the host cell by endocytosis. In general, they become intracellular and surrounded by a layer primarily in a band together with the host plasma membrane. The conclusion is that this is analog to symbiosome membrane enclosing bacteroids in the nodule of rhizobium legume interaction (Parniske, 2000); however, nodules are not formed.

V. CONCLUSION

The research indicates that the determinants of the interaction between the G. diazotrophicus bacteria and spinach seedlings were suitable for the expression of the gene responsible for the formation of the nitrogenase enzyme. G. diazotrophicus lives inside plant tissue cells in the form of colonies and excretes about half of the fixed nitrogen, which offers potential power that improves plant growth.

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