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ASSESSMENT OF *GUS*-LABELED *GLUCONACETOBACTER DIAZOTROPHICUS* INTERACTION WITH SUGAR BEET (*BETA VULGARIS* L.)

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SUMMARY

Based on isolating *Gluconacetobacter diazotrophicus* from the intercellular spaces of aseptically produced sugar beet root (*Beta vulgaris* L.) seedlings in sucrose-containing culture conditions, the following study determined the interaction of *G. diazotrophicus*, a non-nodulating endophytic nitrogen-fixing bacterium. The entire root system gained intracellular colonization by *G. diazotrophicus* after inoculating the plant with the bacterium. Sugar beet seedlings' root tips and cotyledons inoculated with *GUS*-labeled genes bore scrutiny under a light microscope to examine the blue-stained *G. diazotrophicus* in the root cells' cytoplasm. The favorable environment within the cell helped produce the *nitrogenase nif* gene. Novel inoculations with *G. diazotrophicus* underwent investigation for their ability to promote the non-nodular endosymbiotic nitrogen fixation. The inoculations' viability as a plant model for investigating the endosymbiotic theory of organelle generation in eukaryotic organisms is another vital question requiring answers.

Keywords: Sugar beet (*B. vulgaris* L.), *GUS* gene, *G. diazotrophicus*, *nif* nitrogen fixation, interaction, eukaryotic organisms

Key findings: Successful intervention of sugar beet (*B. vulgaris* L.) seedlings with *GUS*-labeled *G. diazotrophicus* occurred for the first time, expressing the possibility of fixing atmospheric nitrogen (N_2) as an alternative to Rhizobium bacteria.

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INTRODUCTION

Sugar beet (*Beta vulgaris* L.) is an important sucrose-producing crop being grown commercially for sugar production in temperate zones. In plant breeding, it is typically the Altissima cultivar group of the common beet (*Beta vulgaris*). Together with other beet cultivars, such as beetroot and chard, it belongs to the subspecies *Beta vulgaris* subsp. *vulgaris*; however, its classification is *var. saccharifera*, and the sea beet (*Beta vulgaris* subsp. *maritima*) is its closest wild relative (Beta Maritima, 2012; Sorting Beta Names, 2013). Sugar beets can grow in climates that are too cold for sugarcane. Russia, the USA, Germany, France, and Turkey are the world's largest sugar beet producers.

More than 16% of global sugar requirements come from sugar beets planted on approximately 4.2 million hectares (Wimmer and Sauer, 2020). Owing to its elevated productivity, sugar beet root is gaining prominence as a sugar source, as well as a potential 'green bioreactor' for accumulation of novel metabolites in its roots (Amo-Mateos *et al.*, 2022). The tissue culture technology application has emerged as successful with sugar beet (Al-Nema and Al-Mallah, 2013) and various other crop and medicinal plants (Al-Nema and Abdullah, 2023).

Gluconacetobacter diazotrophicus, a nitrogen-fixing bacterium known formerly as *Acetobacter diazotrophicus*, had its first discovery in sugarcane roots and stems (Grillo-Puertas *et al.*, 2018; Taher and Saeed, 2023). Numerous essential crops have been observed to contain these endophytic non-rhizobacterial bacteria (Sebring, 2021). A previous study recently identified the said species in *Spinacia oleracea* seedlings (Masyeb *et al.*, 2024). By using $^{15}N_2$ as an incorporation test, the *G. diazotrophicus* bacterium was effective at fixing the nitrogen inside sugarcane plants (Sevilla *et al.*, 2001; Boddey *et al.*, 2003).

GUS refers to a gene reporter system that uses the enzyme *glucuronidase* (*GUS*) to provide insight into gene expression. The *G. diazotrophicus* bacterium can release more than 50% of the fixed nitrogen used by crop plants (Cojho *et al.*, 1993). Moreover, it penetrates the intercellular spaces of root meristems and lateral roots in the sugarcane plants without producing nodules in the xylem. Notably, the *G. diazotrophicus*, diazotrophic endophytic *Azoarcus* spp., *Herbaspirillum* spp., and *Klebsiella pneumoniae* 342 have not shown to colonize live plant cells intracellularly (James *et al.,* 2001, 2002; Iniguez *et al.,* 2004; Rasheed *et. al*., 2024).

A reporter gene is a gene whose protein has a property allowing it to be evident in vitro (fluorescence and detectable enzyme activity). The most valuable types of genes are *GUS*, *NPT* II, *GFP*, *CAT*, and *LUC*. These genes serve to measure the gene expression of target genes by combining them with their regulatory sequences (Miki, 2008). The *GUS* gene was applicable with *G. diazotrophicus* bacteria in detecting its presence in wheat (*Triticum aestivum* cv. baguette) and sorghum (*Sorghum bicolor* L.) (Luna *et al.*, 2010) and spinach seedlings (Younis and Saeed, 2023; Masyeb *et al.,* 2024).

Results about this gene with other types of bacteria interfering with plants will play a vital role because of its diagnostic importance in distinguishing the interaction between bacteria and crop plants. One study succeeded in monitoring the *GUS* gene in transformed hairy roots cultures in tomato and potato using the X-Gluc dye. Its blue color was evidence of the gene's transfer with the genes of *Agrobacterium rhizogenes* to the plant's genome inoculated with this bacterium (Al-Mallah and Masyeb, 2014). Based on the above discussion, the presented study sought to investigate an efficient protocol for the interaction of *GUS*-labeled *G. diazotrophicus* with sugar beet seedlings.

MATERIALS AND METHODS

Seed germination and plant culture

Seed samples of sugar beet (*Beta vulgaris* var. Baraka) came from the General State of Producing Sugar, Mosul, IRAQ. The seeds were surface sterilized using appropriately diluted 'Domestos' bleach, containing 5% sodium

hypochlorite (Lever Fabergé, Kingston-upon-Thames, UK), and subsequently rinsed in sterile water. Surface-sterilized sugar beet seeds' cultivation in autoclaved vermiculite and sterile water reached a period of five days at 28 °C under a 16-h photoperiod. After five days, transferring seedlings (roots with 1.0 cm in length) proceeded into sterile agar-solidified (MSO) MS hormone-free medium (Murashige and Skoog, 1962) in tiny jars, keeping one seedling per jar. The seedlings' maintenance in the growth chamber had a temperature of 25 °C with light for four days to assess their growth.

Culture of *GUS*-labeled *G. diazotrophicus*

The growing of *G. diazotrophicus* UAP5541 strain (Fuentes-Ramirez *et al*., 1999), which constitutively expresses *GUS*, transpired on an AT-*GUS* medium. The medium consisted of 0.8% (w/v) agar, 2.7 g L^{-1} yeast extract, glucose (2.7 g L^{-1}), mannitol (1.8 g L^{-1}), MES buffer (4.4 g L^{-1}), and potassium bicarbonate (0.65 g L^{-1}), and adjusting to a pH of 6.5, to meet the prescribed streptomycin concentration of 45 mg L^{-1}. The X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid cyclohexylammonium salt) addition to AT-*GUS* medium had a concentration of 50 mg L^{-1} to assess the expression of the *β-glucuronidase* (*GUS* A) gene. A gene expression signal known as *GUS* A was notable when dark blue colonies emerged (Cocking *et al.,* 2006).

Inoculation procedure

The goal of developing an aqueous *G. diazotrophicus* suspension was to reach an optical density of 600 nm of 1.1 × 10^9 colony-forming units (CFU ml^{-1}). Using the AT-*GUS* medium helped determine the CFU count by serial dilution and plating. Counting bacterial colonies in Petri plates continued for four days in a dark environment at 28 °C. According to Cocking *et al.* (2006), the last dilution (10^9) succeeded in inoculating 25 seedlings whose root system was present in MS medium by adding 1.0 ml of the suspension to them, while giving other seedlings 1.0 ml of sterile water as a control.

Histochemical staining using X-Gluc

The light microscopy usage helped examine the dark blue precipitate produced when the *β-glucuronidase* broke down X-Gluc, allowing for the observation of bacterial colonizations. After removing any surplus agar, removal of plants infected with *G. diazotrophicus* and control plants ensued from the medium. Using dimethyl formamide instead of dimethyl sulfoxide without $Na_2S_2O_5$, the histochemical staining followed the steps previously described (Fuentes-Ramirez *et al.,* 2001). Before being treated overnight at 37 °C with X-Gluc staining solution, the samples sustained vacuum infiltration for 30 min. Before analysis, the samples underwent three rounds of washing in a 0.1 M sodium phosphate buffer (pH 7.0). The samples reached fixing in a solution containing 2% glutaraldehyde by volume using a water-based fixative.

Direct microscopic observations

At each sampling interval, roots and cotyledonary leaves' removal from the plants histochemically stained with X-Gluc occurred and underwent examination using brilliant field microscopy. The scrutiny of root and cotyledon leaf portions continued with samples demonstrating blue *GUS* activity. The specimens' fixing employed 2% glutaraldehyde in 0.1 M sodium phosphate buffer for 24 h at 4 °C and subsequently dehydrated with ethanol. The implanted medium-grade acrylic resin in white (Agar Aids, UK) sectioned to a thickness of approximately 1.4 µm, counterstained with safranin (0.01%), bore assessment with 10× and 100× objectives. The repetition of study (histochemical staining and observation) proceeded in a 12-day post-inoculation.

RESULTS

Seedling inoculation with *GUS-G. diazotrophicus*

After inoculating sugar beetroot seedlings with *G. diazotrophicus*, the conditions facilitating intracellular colonization of root tips were

noteworthy. All the studied samples exhibited he most significant root and shoot development in agar-solidified MS media. Inoculation of juvenile seedlings cultivated on MS-agar medium proceeded with 10^9 CFU. This number was typically applicable for inoculating sugar beet seedlings with the *G. diazotrophicus* strain UAP5541.

Chemical detection of bacteria using X-Gluc

The visual observations revealed the X-Gluc dye solution transformed from colorless to blue in infected seedlings after 24 h, whereas it remained colorless in uninoculated seedlings. Furthermore, the leaves of infected sugar beet seedlings exhibited a blue coloration in contrast to the leaves of uninoculated seedlings.

Bacterial presence in tissues

The bacterial presence was evident with two stages:

a) Roots and cotyledonary leaves with 4d post-inoculation

Microscopic examination of root tips exhibited the presence of bacterial cells in the cell wall of these roots (Figures 1A and B), while bacterial cells were absent in the root tips of control samples of sugar beet (Figures 1C and D). The results also proved the presence of bacterial cells in the cell wall of epidermal cells of cotyledonary leaves (Figure 2A), and some sections also showed the bacterial cells displayed a close association with vesicles (Figure 2B). In other sections, the viewed bacterial cells stationed near the cell walls of guard cells (Figure 2C), and they were absent in control samples (Figure 2D).

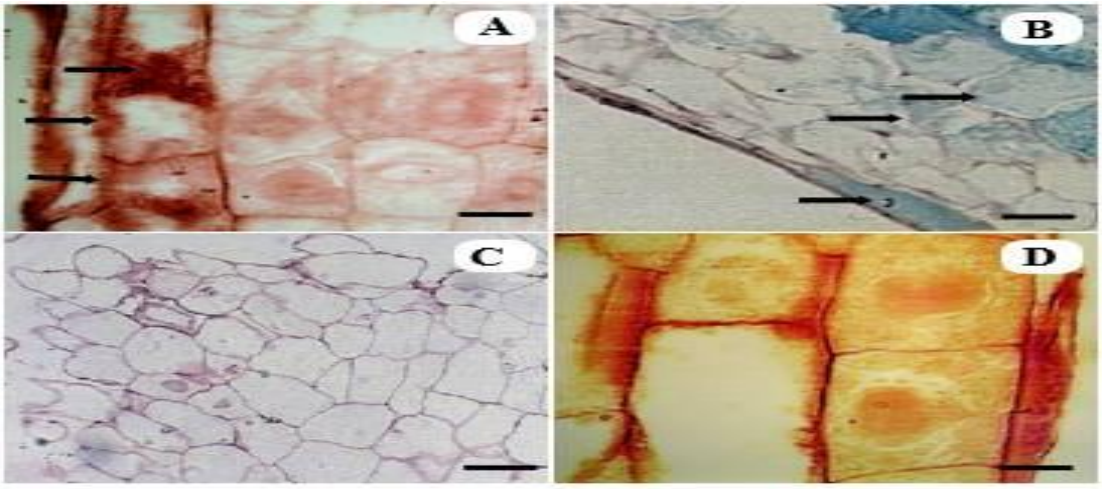

Figure 1. Light micrographs of roots of *Beta vulgaris* L. seedlings inoculated with *G. diazotrophicus* four days after inoculation (A–B) and uninoculated control (C–D). (A) Section of root inoculated with *GUS*-A *G. diazotrophicus* showing bacteria within cells and in cell walls (arrows). (B) Section of root four days after inoculation with *GUS*-A *G. diazotrophicus*, showing more extensive intracellular colonization including the presence of microcolonies (arrows). (C) Longitudinal section of histochemically stained root tip of uninoculated (control). (D) Section of histochemically stained peripheral cells of root tip of uninoculated (control). Scale bars ¼ = 10 mm**.**

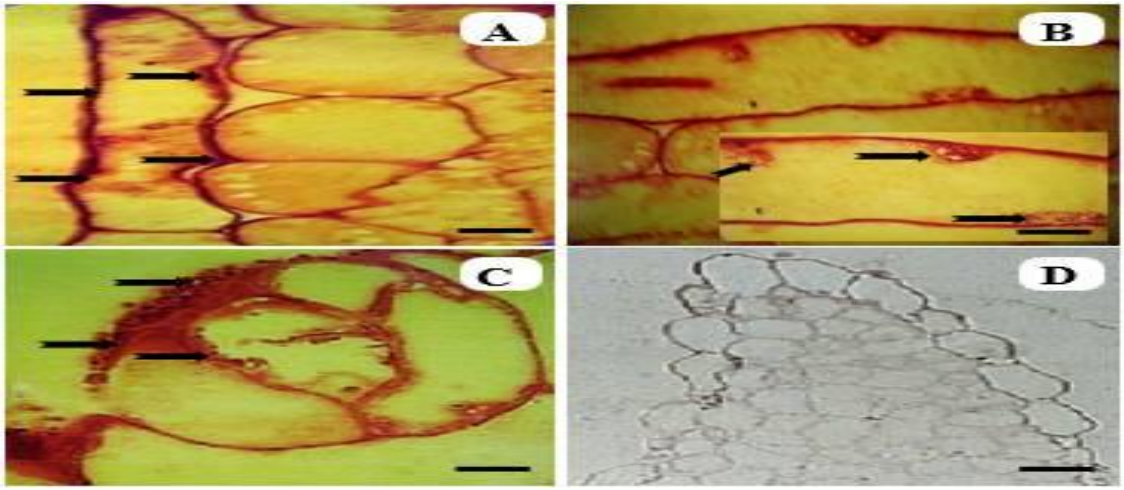

Figure 2. Light micrographs of cotyledon leaves from *Beta vulgaris* L. seedlings, inoculated with *G. diazotrophicus* four days post-inoculation (A–C), alongside the uninoculated control (D). Portion of leaf infected with *GUS–G. diazotrophicus*, exhibiting bacteria within cells and in cell walls (shown by arrows). (B) Leaf slice four days post-inoculation with *GUS–G. diazotrophicus*, displaying elongated pleomorphic bacteria and substantial intracellular colonization of leaf cells, with bacteria closely associated with vesicles (arrows). Bacterial cells were aggregated along the cell walls of guard cells (arrows). Histochemically stained slice of the cotyledon from uninoculated control specimens. Scale bars ¼ = 10 mm.

b) Roots and cotyledonary leaves with 12-day post-inoculation

After 12 days of inoculation, the portions of roots and cotyledon leaves fixed with resin, and the *GUS-G. diazotrophicus* inoculation revealed the presence of *diazotrophicus* inside cells dyed dark blue (Figures 3A and B). Figures 3C and D showed the invasion and heavy spread of bacteria into the elongation zone. Histochemically stained slices of uninoculated (control) root tips have not exhibited the dark blue-stained *G. diazotrophicus* (Figure 3E).

Colonization inside leaf cells

Under the microscope, the cells with blue-stained *G. diazotrophicus* could be seen (Figure 4A). Additionally, the bacterial cells were also visible within the protective cells (Figures 4B and C). The histochemically stained slices of uninoculated (control) leaves do not contain the dark blue-stained *G. diazotrophicus* (Figure 4D).

DISCUSSION

The growth conditions and low inoculation levels on sucrose-enriched MS-agar medium proved to be ideal for *G. diazotrophicus* and sugar beet plants. This allowed for the exchange of signals requiring for the bacteria to penetrate plant cell walls and colonize within cells. It was likely that one factor was the production of indole acetic acid (IAA) by *G. diazotrophicus.* This compound may hinder plant growth with its high concentrations, as also reported by Lee *et al.* (2004). In bacterial-plant interactions, IAA may function as a signaling molecule at a low concentration (Keswani *et al.,* 2020).

Plants can respond positively and negatively to IAA generated by the microbes. However, the magnitude of IAA found in the plant's roots internally determines its role. A recent study also mentioned the possibility of *G. diazotrophicus* colonizing vesicles inside cells after penetrating their cell wall with sucrose-induced endocytosis (Ganesh *et al.,* 2024). According to Meneses *et al.* (2011), *G.*

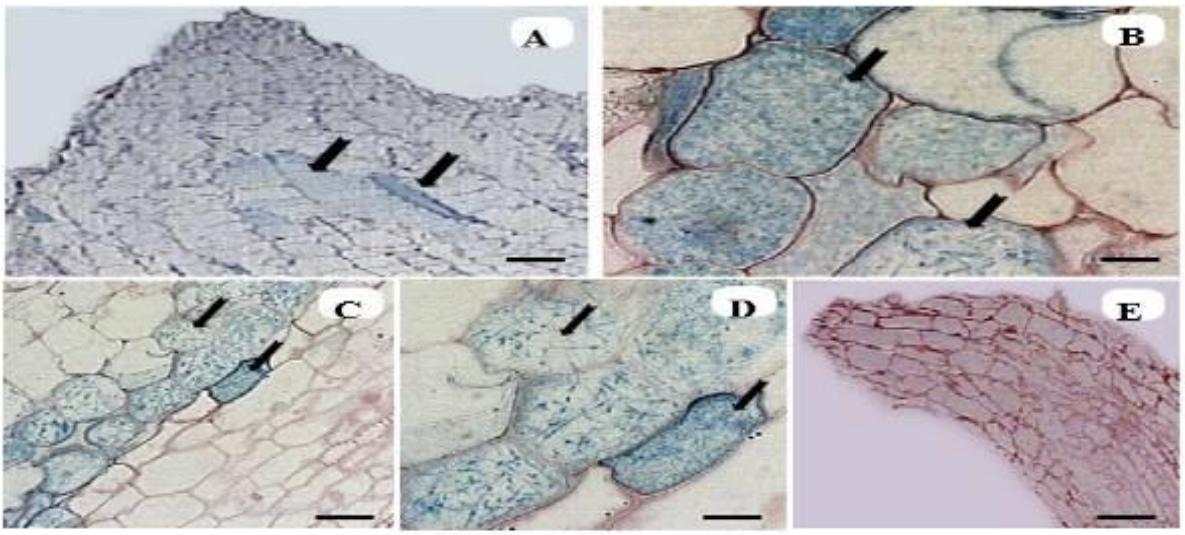

Figure 3. Light micrographs of roots of *Beta vulgaris* L. inoculated with *G. diazotrophicus* 12 days after inoculation (A–D) and uninoculated control (E). (A) Bacteria inside cells are shown in the section of the root infected with *GUS-G. diazotrophicus*. (B) A root section taken 12 days after being inoculated with *GUS-G. diazotrophicus* reveals microcolonies and enhanced intracellular colonization (arrows). In C and D, blue-stained *G. diazotrophicus* (arrows) is shown colonizing the root intracellularly. Part of the root tips of the control group not infected and stained histologically (E). Every 10 millimeters, the scale bars are visible.

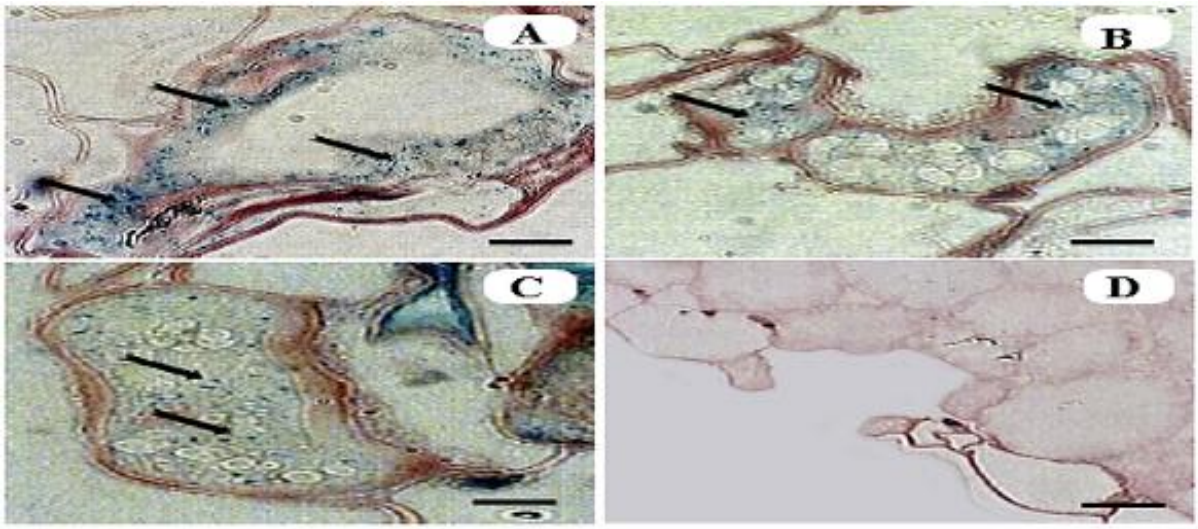

Figure 4. Light micrographs of cotyledon leaves of *Beta vulgaris* L. inoculated with *G. diazotrophicus* 12 days post-inoculation (A–C) and uninoculated control (D). Intracellular colonization of leaf exhibiting blue-stained *G. diazotrophicus* within cells (arrows). Bacterial cells (B, C) invaded guard cells (shown by arrows). Section of histochemically stained cotyledon from uninoculated (control) specimens. Scale bars ¼ =10 mm (A–D).

diazotrophicus also synthesizes exopolysaccharides that help mucoid bacteria to grow. The significant staining may gain further amplification by the *GUS*-histochemical reaction's byproducts (Ruijter *et al.,* 2003) and rarely by mistaken localization (Dedow *et al.,* 2022).

Nitrogen fixation could occur over a broad range of oxygen levels because the *G. diazotrophicus* population uses this mucoid matrix as a considerable barrier against oxygen transport (Pan and Vessey, 2001; Dong *et al.,* 2002). Factually, *G. diazotrophicus* can produce blue-stained bacteria due to its *GUS* gene and *nifH* promoter-*GUS A* combination, which suggests the internal environment of the said plant species supports the development of *nitrogenase* genes. By inoculating the spinach seedlings, the *G. diazotrophicus nifH-GUSA* has the same construction (Masyeb *et al.,* 2024).

The exact intracellular role of a similar oxygen diffusion resistance in *G. diazotrophicus* inside this plant's roots is still a mystery. The further understanding of how exopolysaccharides contribute to the development of *G. diazotrophicus* and if the expression of the *nifH* gene has linkage with exopolysaccharide synthesis requires future studies using mutants that are unable to produce these sugars (Sevilla and Kennedy, 2000; Dietz, 2022). According to Muthukumarasamy *et al.* (2002), the presence of intracellular *G. diazotrophicus*, which has elongated pleomorphic cells, suggests that it thrives in MS media containing the highest concentration of nitrogen sources (NH_4 and NO_3).

Nitrogen fixation and growth mostly occur at a pH of 3.0 and below, demonstrating *G. diazotrophicus's* crucial acid tolerance. *Nitrogenase* activity is being unaffected by 10 mM nitrate, and it does not include *nitrate reductase* (Grillo-Puertas *et al.,* 2018). Additionally, NH_4 induces a limited inhibition of *nitrogenase* (Medeiros *et al.,* 2006). However, striking parallels exist between the angiosperm *Gunnera* and the nitrogen-fixing cyanobacterium Nostoc, as well as the interaction between the non-rhizobial *G. diazotrophicus* and the plant root tips (Santi *et al.,* 2013). To enter the stem's thin-walled meristematic gland cells, the cyanobacteria first break down their cell walls. Then, the host cell uses endocytosis to transfer the bacteria into vesicles, become intracellular, and initially get trapped by a membrane found within the host plasma membrane (Al-Nema *et al*., 2022). Although the roots do not produce nodules, this is similar to the symbiosome membrane that encases bacteroids in the *Rhizobium*-legume symbiosis (Parniske, 2000).

CONCLUSIONS

Successful intervention of sugar beet seedlings with *GUS*-labeled *Gluconacetobacter diazotrophicus* proceeded for the first time, expressing the possibility of fixing atmospheric nitrogen N_2 as an alternative to Rhizobium bacteria. The efficiency of the *GUS* protocol, as also assessed, was valid in detecting the interaction of sugar beet with the *GUS*-labeled bacteria *G. diazotrophicus*.

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