http://dx.doi.org/10.14500/aro.11239

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

Hikmat M. Masyeb[1], Qutaiba S. Al-Nema[2] and Mozahim K. Al-Mallah[3,*]

[1]Department of Biology, Faculty of Science and Health, Koya University, Koya KOY45, Kurdistan Region - F.R. Iraq

[2]Department of Biology, College Education for Pure Sciences, University of Mosul, Mosul, F.R. Iraq

[3]Department of MLT, Al-Hadbaa University College, Mosul, F.R. Iraq

***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

ARO-The Scientific Journal of Koya University
Vol. XII, No. 1 (2024), Article ID: ARO.11239. 5 pages
DOI: 10.14500/aro.11239
Received: 07 July 2023; Accepted: 20 December 2023
Regular review paper: Published: 08 January 2024
Corresponding author’s e-mail: hikmat.mustafa@koyauniversity.org

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 (10^9) 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

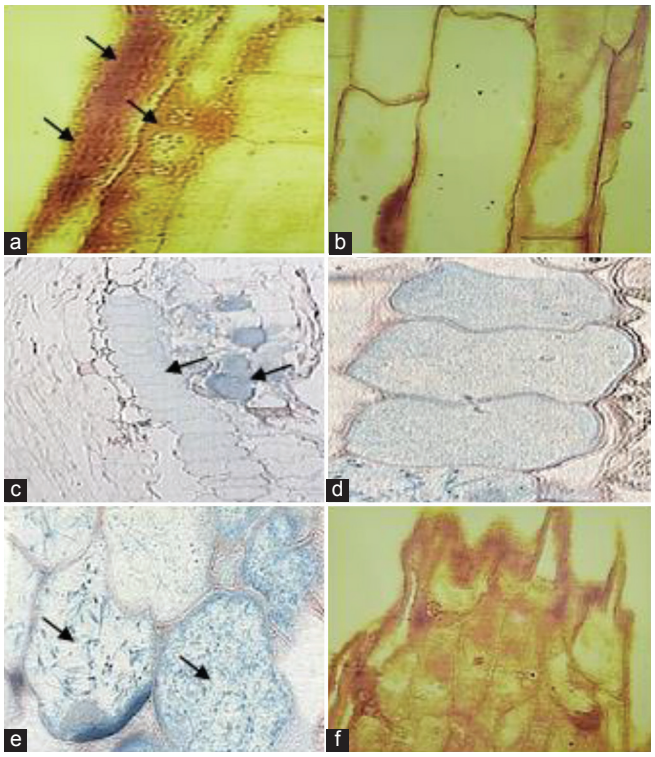

Fig. 1. Illumination micrographs of roots of *Spinacia oleracea* L. inoculated with *Gluconacetobacter diazotrophicus* 4 days and 12 days after inoculation and un-inoculated control. (a) Root section inoculated through *GUS*A–*G. diazotrophicus* viewing bacteria inside the cells and within the walls (arrows) 4 days after inoculation. (b) Longitudinal section of the stained un-inoculated root tip (control). (c) Root section inoculated through *GUS*A–*G. diazotrophicus* screening bacteria inside the cells (arrows) 12 days after inoculation. (d and e) Section of root inoculated through *GUS*A–*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).

http://dx.doi.org/10.14500/aro.11239

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 *GUS*A 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 and Tian, 2014). 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_4NO_3, NH_4Cl, KNO_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).

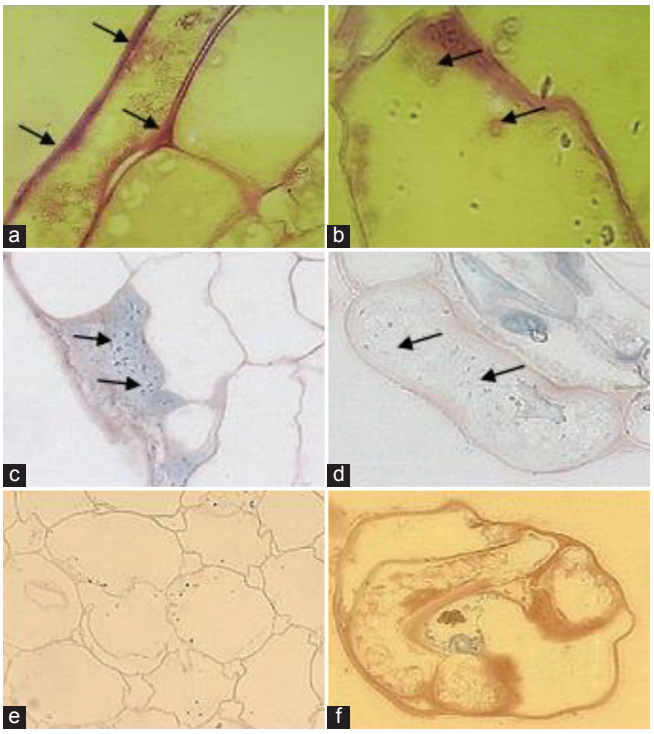

Fig. 2. Illumination micrographs of cotyledon leaves for *Spinacia oleracea* L. inoculated with *Gluconacetobacter diazotrophicus* 4 days and 12 days after inoculation and un-inoculated control. (a) Inoculated leaf section through *GUS*A–*G. diazotrophicus* viewing bacteria inside the cells beside the cell walls (arrows). (b) Leaf section at 4 days after inoculation through *GUS*A–*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 (NH_4) 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.

Acknowledgment

The authors would like to thank Professor E. C. Cocking, School of Biology, and Nottingham University, UK, for supplying *G. diazotrophicus* bacteria. Once again, the authors are very grateful to the Department of Biology, Faculty of Science and Health, Koya University and University of Mosul/College of Education for Pure Sciences, and Al-Hadba University Colleges for their facilities that sustained this investigation.

References

AL-Mallah, M.K., and Masyab, H.M., 2014. Expression of GUS and GFP reporter genes in transgenic hairy roots of tomato and potato plants via *Agrobacterium rhizogenes* mediated transformation. *Australian Journal of Basic and Applied Sciences*, 8(2), pp.234-239.

Boddey, R.M., Urquiaga, S., Alves, B.J.R., and Reis, V., 2003. Endophytic nitrogen fixation in sugarcane: Present knowledge and future applications. *Plant and Soil*, 252, pp.139-149.

Chiu, W., Peters, G.A., Levieille, G., Still, P.C., Cousins, S., Osborne, B., and Elhai J., 2005. Nitrogen deprivation stimulates symbiotic gland development in *Gunnera manicata*. *Plant Physiology*, 139, pp.224-230.

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 Cellular and Developmental Biology-Plant*, 42, pp.74-82.

Dietz, B.R., 2022. *Genetic Manipulations in Known Endophytes Furthering the Application as a Biofertilizer. M. Sc. Thesis*. University of Minnesota, Minnesota, U.S.A.

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 protection of nitrogenase from O_2 by colony structure in the aerobic diazotroph *Gluconacetobacter diazotrophicus*. *Microbiology* (*Reading*), 148, pp.2293-2298.

Duca, D., Lorv, J., Patten, C.L., Rose, D., and Glick, B.R., 2014. Indole-3-acetic acid in plant-microbe interactions. *Antonie van Leeuwenhoek*, 106, pp.85-125.

Eskin, N., Vessey, K., and Tian, L., 2014. Research progress and perspectives of nitrogen fixing bacterium, *Gluconacetobacter diazotrophicus*, in monocot plants. *International Journal of Agronomy*, 2014, p.208383

Furness, D., Fenech, M., Dekker, G., Khong, T.Y., Roberts, C., and Hague, W., 2013. Folate, Vitamin B12, Vitamin B6 and homocysteine: Impact on pregnancy outcome. *Maternal and Child Nutrition*, 9(2), pp.155-166.

Grillo-Puertas, M., Delaporte-Quintana, P., Pedraza, R.O., and Rapisarda, V.A., 2018. Intracellular polyphosphate levels in *Gluconacetobacter diazotrophicus* affect tolerance to abiotic stressors and biofilm formation. *Microbes and Environment*, 33(4), pp.440-445.

Kong, Q., Li, J., Wang, S., Feng, X., and Shou, H., 2023. Combination of hairy root and whole-plant transformation protocols to achieve efficient CRISPR/Cas9 genome editing in soybean. *Plants* (*Basel*), 12, p.1017.

Lambrecht, M., Okon, Y., Vande Broek, A., and Vanderleyden, J., 2000. Indole-3-acetic acid: A reciprocal signalling molecule in bacteria-plant interactions. *Trends Microbiology*, 8, pp.298-300.

Masyab, H.M., 2018. Expression patterns of *GUS* gene in five different strains of *Arabidopsis thaliana* plants. In: *International Conference on Pure and Applied Sciences (ICPAS 2018)*, pp.59-63.

Medeiros, A.F.A., Polidoro, J.C., and Reis, V.M., 2006. Nitrogen source effect on *Gluconacetobacter diazotrophicus* colonization of sugarcane (*Saccharum* spp.). *Plant and Soil*, 279(1-2), pp.141-152.

Miki, B., 2008. Marker genes and promoters. In: Stewart, C.N., editor. *Plant Biotechnology and Genetics: Principles, Techniques, and Applications*. John Wiley and Sons, Ltd., New Jersey, U.S.A.

Mohammed, A.A.H., and Masyab, H.M., 2020. Genetic transformation of *Nigella sativa* L. Plants with *Agrobacterium rhizogenes* 35S GUS R1000 and estimation of thymoquinone level in transformed hairy roots cultures. *Plant Archives*, 20(Suppl 1), pp.3649-3652.

Murashige, T., and Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue culture. *Physiologia Plantarum*, 15, pp.473-497.

Muthukumarasamy, R., Revathi, G., and Loganathan, P., 2002. Effect of inorganic N on the population, *in vitro* colonization and morphology of *Acetobacter diazotrophicus* (syn. *Gluconacetobacter diazotrophicus*). *Plant and Soil*, 243, pp.91-102.

Parniske, M., 2000. Intracellular accommodation of microbes by plants: A common developmental program for symbiosis and disease. *Current Opinion in Plant Biology*, 3, pp.320-328.

Rasheed, J.H., 2014. *GUS*-gene as a visual marker for *Gluconacetobacter diazotrophicus* co-cultivated with carrot plantlets. *International Journal of Science and Technology*, 3, pp.776-779.

Rocafull, Y.R., Badia, M.J., Garciea, M.O., Álvarez, B.D., and Sánchez, J.R., 2016. Isolation and characterization of *Gluconacetobacter diazotrophicus* strains. *Cultivos Tropicales*, 37(1), pp.34-39.

Rodriguez, M.V., Tano, J., Ansaldi, N., Carrau, A., Srebot, M.S., Ferreira, V., Martínez, M.L., Cortadi, A.A., Siri, M.I., and Orellano, E.G., 2019. Anatomical and biochemical changes induced by *Gluconacetobacter diazotrophicus* stand up for *Arabidopsis thaliana* seedlings from *Ralstonia solanacearum* infection. *Frontiers in Plant Science*, 10, p.1618.

Ruhullah, M., Rahman, M.S., Sarafat, A.M.D., Mohsin G.M., and Khalekuzzaman, M., 2017. Establishment of agrobacterium mediated genetic transformation in popular rice cultivar BRRI dhan-29 through reporter GUS gene expression system in Bangladesh. *FPI*, 1(2), pp.67-75.

Sebring, R.L., Duiker, S.W., Berghage, R.D., Regan, J.M., Lambert, J.D., and

Bryant, R.B., 2022. *Gluconacetobacter diazotrophicus* inoculation of two lettuce cultivars affects leaf and root growth under hydroponic conditions. *Applied Sciences*, 12, p.1585.

Sevilla, M., and Kennedy, C., 2000. Genetic analysis of nitrogen fixation and plant growth stimulating properties of *Acetobacter diazotrophicus*, an endophyte of sugarcane. In: Triplett, E.W., editor. *Prokaryotic Nitrogen Fixation: A Model System for Analysis of a Biological Process*. Scientific Press, Wymondham, UK, Horizon, pp.737-760.

Shojaei, T.R., Salari, V., Ramazan, D., Ehyaei, M., Gharechahi, J., and Chaleshtori, R.M., 2010. The effect of plant growth regulators, explants and cultivars on spinach (*Spinacia oleracea* L.) Tissue culture. *African Journal of Biotechnology*, 9(27), pp.4179-4185.

Varghese, F., Kabasakal, B.V., Cotton, C.A.R., Schumacher, J., Rutherford, A.W., Fantuzzi, A., and Murray, J.W., 2019. A low-potential terminal *oxidase* associated with the iron-only *Nitrogenase* from the nitrogen-fixing bacterium *Azotobacter vinelandii*. *Journal of Biological Chemistry*, 294(24), pp.9367-9376.

Xiong, A.S., Peng, R.H., Zhuang, J., Chen, J.M., Zhang, B., Zhang, J., and Yao, Q.H., 2011. A thermostable β-glucuronidase obtained by directed evolution as a reporter gene in transgenic plants. *PLoS One*, 6(11), e26773.