

Growth of *Cicer arietinum* With *Azotobacter vinelandii* and *Burkholderia vietnamiensis* Endophytes of *Leucaena leucocephala* and 50% NH_4NO_3

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Abstract

Agricultural production of *Cicer arietinum* demands, the use of nitrogen fertilizer as NH_4NO_3 , its excessive application causes the loss of soil productivity and global warming by releasing N_2O . An ecological to solve this problematic condition could be: to isolate endophytic bacterial genera that promote plant growth such as: *Azotobacter vinelandii* and *Burkholderia vietnamiensis*, from *Leucaena leucocephala* a wild plant able to face nutritional environmental, that allow reducing and optimizing the dose of NH_4NO_3 at 50%, non-compromising healthy plant growth of *C. arietinum*. Therefore, the objectives of this work were: i) to isolate of endophytic genus of *A. vinelandii* and *B. vietnamiensis* from *L. leucocephala*, ii) to analyze the effect of *A. vinelandii* and/or *B. vietnamiensis* on growth of *C. arietinum* at 50% NH_4NO_3 , iii) to identify *A. vinelandii* and *B. vietnamiensis* based on the biochemical profile. The isolation of *A. vinelandii* was in Burk agar and *B. vietnamiensis* on PCAT. The results showed that *A. vinelandii* and *B. vietnamiensis* isolated from *L. leucocephala* were beneficial to the healthy growth of *C. arietinum* by increasing the germination percentage and reducing the emergence time while both the phenology and biomass of *C. arietinum* were positively affected by 50% NH_4NO_3 ; based on the conversion of organic compounds in the root into phytohormones by *A. vinelandii* and/or *B. vietnamiensis* better when inoculated as a mixture than individually with numerical values that were statistically different by ANOVA-Tukey ($P < 0.05$), compared to the same numerical variables of *C. arietinum* growth with 100% NH_4NO_3 uninoculated, used as a relative control. While it was found that *A. vinelandii* showed a biochemical profile of this genus and species with cyst production while *B. vietnamiensis* had the biochemical profile of this genus and species. The above supports the idea that *L. leucocephala* is an important source of plant growth-promoting endophytic bacteria for sustainable agriculture without damaging the soil or generating N_2O to mitigate due to conventional agriculture production of *C. arietinum*.

Keywords: agriculture, legumes, soil, greenhouse gases, colonization, root system, biofertilizer, phytohormones

1. Introduction

The cultivation of *Cicer arietinum* (chickpea) is crucial for food security in many parts of the world, especially in regions where it serves as a primary source of protein in the diet. Traditionally, *C. arietinum* cultivation has relied on the application of synthetic nitrogen fertilizers, such as ammonium nitrate (NH_4NO_3), to ensure adequate nitrogen supply for optimal growth. However, excessive fertilizer use can lead to environmental

concerns such as soil degradation, contamination of surface water and aquifers, and the emission of nitrous oxide (N_2O), a potent greenhouse gas. Furthermore, nitrogen fertilization does not always result in expected crop yields, particularly under suboptimal soil conditions or when the plant is unable to effectively utilize the applied nutrients (Anjum & Chandra, 2015; Bhattacharya, 2019).

In conventional, protected, and organic agricultural systems, the application of various *Rhizobium* species is a common practice to enhance nitrogen fixation in leguminous plants like *C. arietinum*. However, these responses are often inconsistent, particularly when soil fertility is low or other environmental stressors are present (Chowdhury-Paul et al., 2018). One promising approach to overcome these limitations is the use of alternative microbial inoculants—specifically endophytic bacteria—that can promote plant growth and improve nutrient uptake. Recent studies have shown that endophytic species can enhance nitrogen fixation, increase nutrient availability, and improve plant resilience to environmental stresses (Guo et al., 2016, 2020).

One such potential source of beneficial endophytic bacteria is *Leucaena leucocephala*, a leguminous species known for its ability to fix atmospheric nitrogen and enrich the soil with organic matter. As a green manure and forage crop, *L. leucocephala* not only provides biomass for improving soil health but also hosts a variety of plant growth-promoting bacteria (PGPB) within its root system. Among the diverse genera of PGPB, *Azotobacter vinelandii* and *Burkholderia vietnamiensis* have garnered attention due to their ability to promote nitrogen fixation, increase plant growth, and outcompete less beneficial microbial species (Martínez-Ortiz et al., 2020; Marentes-Culma et al., 2022;). These bacteria have shown promise as biofertilizers when used individually or in combination, potentially reducing the need for synthetic nitrogen inputs while enhancing the plant's nutrient uptake efficiency.

By inoculating *C. arietinum* with these beneficial endophytic bacteria, it may be possible to significantly reduce the dependency on high doses of nitrogen fertilizers such as NH_4NO_3 , while also improving soil health and reducing the environmental impact of agricultural practices. Additionally, incorporating a mixture of bacteria with competitive capacity could enhance the symbiotic relationship between the plant and microorganisms, facilitating more efficient nitrogen uptake and minimizing nutrient losses (Raiten & Aimone, 2017; Rai et al., 2020;). Moreover, such an approach may help prevent the accelerated loss of soil organic matter, reduce contamination of surface waters and aquifers, and mitigate the generation of N_2O —a greenhouse gas commonly associated with excess nitrate in agricultural systems.

The objectives of this study are: i) to isolate the endophytic bacteria *Azotobacter vinelandii* and *Burkholderia vietnamiensis* from *L. leucocephala*; ii) to assess the effect of these bacteria, in combination with 50% NH_4NO_3 , on the growth and nitrogen uptake of *C. arietinum*; and iii) to identify the bacterial strains based on their biochemical profiles, to better understand their potential as effective biofertilizers.

2. Material and Methods

2.1 Origin of *A. vinelandii* and *B. vietnamiensis*

The isolates of *A. vinelandii* and *B. vietnamiensis* were isolated from inside roots of *L. leucocephala*, grown for 45 days in pots with sodium lateritic soil poor in organic matter and mineral nitrogen (N) from Morelia, Mich, México in a greenhouse in the Environmental Microbiology Laboratory of the Institute Research Chemistry Biology of the UMSNH. The root of *L. leucocephala* was cut from the crown part, from there the soil was removed with tap water in a laminar flow the root was cut 5 cm long with scissors, disinfected with 3% sodium hypochlorite (v/v), then with 70% alcohol (v/v); following the root was rinsed 6 times with sterile water, then with 70% alcohol (v/v)/5 min and washed 6 times with sterile water; then 2 cm of the root was suspended in 5 mL of 0.85% (w/v) NaCl saline solution in a sterile mortar, where it was crushed, from this suspension 0.5ml was sown in Burk broth (g/L): glucose 10.0; K_2HPO_4 2.0; KH_2PO_4 2.0; MgSO_4 3.0; a trace element solution was added 1 mL/L of culture medium, with the following composition (g/L): H_3BO_3 2.86; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.22; $\text{MnCl}_2 \cdot 7\text{H}_2\text{O}$ 1.81; K_2MnO_4 0.09 at pH 6.4-6.7; bromothymol blue 1% (w/v) 10 mL/L of culture medium, the pH was adjusted 6.5-6.7 for *A. vinelandii* and the growth of fungi was inhibited with the antifungal Tecto 60® (Syngenta) 2% (w/v) 1.0 mL/L of culture medium. For recovering *B. vietnamiensis* from suspension of *L. leucocephala* was used PCAT broth: *Pseudomonas cepacia* azaleic acid and tryptamine (g/L): tryptamine 0.2 that was dissolved in a test tube with 2 mL of 70% alcohol, 2 g azaleic acid was dissolved in a 18 × 100 test tube with 2 mL of 70% alcohol: g/L: K_2HPO_4 4.0; yeast extract 0.02; MgSO_4 0.2, the pH was adjusted to 6-6.5 and added the antifungal Tecto 60® (Syngenta) 2% (w/v) 1.0 mL/L of culture medium was also used, the PCAT and Burk tubes were incubated at 30°C/24 h when turbidity was detects as a measure of growth, besides that Gram stain in the case of *A. vinelandii*, the detection of cysts; for this, this isolate on Burk agar with butyl alcohol (g/L): 1% butyl alcohol (v/v) 10.0 mL/L; K_2HPO_4 2.0; KH_2PO_4 2.0; MgSO_4 3.0; trace element solution 1 mL/L; 1% bromothymol blue

10 mL/L; agar 18; at pH 6.5-6.7, incubated at 30 °C/1 week, then staining was performed to observe the formation of cysts under the Carl Zeiss microscope: a portion of the culture was placed on a slide and 2 drops of Lugol's iodine were added/1 min, it was washed and its existence was observed (Raiten & Aimone, 2017; Rana et al., 2021). Gram staining was performed to observe the morphology under the Carl Zeiss microscope: *A. vinelandii* as a large Gram-negative rod, it was purified in Petri dishes with Burk agar and 18 g/L of bacteriological agar, while in the PCAT broth, short Gram-negative rods were observed, which ones were reseeded in PCAT for this, 15 g/L of bacteriological agar was added. Both Petri dishes of Burk agar and PCAT were incubated at 30 °C/48-72 h, to obtain the axenic cultures that were preserved in sterile agricultural soil.

2.2 Assay to Inoculate *A. vinelandii* and *B. vietnamiensis* in *Cicer arietinum* With 50% NH_4NO_3

Then disinfected seeds of *C. arietinum* were inoculated with *A. vinelandii* and *B. vietnamiensis* to analyze the effect on germination to seedling was registered with 50% NH_4NO_3 based on phenology and biomass with a block design compared to *C. arietinum* with 100% NH_4NO_3 uninoculated for 30 days (Chowdhury-Paul et al., 2018; Martínez-Ortiz et al., 2020; Marentes-Culma et al., 2022) results were analyzed by ANOVA-Tukey ($P < 0.05$).

2.3 Biochemical Identification of *A. vinelandii* and *B. vietnamiensis*

Subsequently, biochemical tests were performed to identify each one to corroborate the genus and species of *A. vinelandii* comparative reference for identifying *A. vinelandii*: *A. vinelandii* WU-1 donated by Dr. J. Sadoff of Michigan State University, Michigan, USA, *A. vinelandii*-UW, donated by Dr. J. Page of the University of Alberta, Canada, and *A. vinelandii* 2489, donated by Dr. J. Moreno of the University of Granada, Spain, were used. For the induction of *Azotobacter* cysts, strains were plated on Burk agar in 0.3% (v/v) n-butanol as a glucose substitute, while a strain of *B. vietnamiensis* endophyte from *Oryza sativa* donated by Dr Tran Van V of Microbial Ecology laboratory at Claude Bernard University, Lyon, France was used as reference to identify our isolate from *L. leucocephala* (Rangjaroen et al., 2015; Rai et al., 2020; Rana et al., 2020a;).

3. Results and Discussion

Figure 1 shows the effect of individual *A. vinelandii* or *B. vietnamiensis* with 50% NH_4NO_3 on *C. arietinum* germination that was statistically different from that observed with 100% NH_4NO_3 , while the mixture of both *A. vinelandii* and *B. vietnamiensis* with 50% NH_4NO_3 was statistically different from the seed used as a relative control with 100% NH_4NO_3 not inoculated. Evidence of the effect of *A. vinelandii* or *B. vietnamiensis* that were absorbed by the seed where they increased the synthesis of phytohormones associated with germination thus increasing the activity of amylase for greater germination was especially evident that the combination of both genera and species of endophytes reduced the germination time and the germination percentage to the maximum (Rangjaroen et al., 2015; Rawat, Sanwal, & Saxena, 2018; Rana et al., 2020b). These facts demonstrate that the endophytic, activity of *A. vinelandii* and/or *B. vietnamiensis* is an option for the production of *C. arietinum* uninoculated by *Rhizobium*, saving the NH_4NO_3 dose by 50%, that reduces N_2O generation compared to what happens when the seed is not inoculated with these endophytes and the maximum dose of NH_4NO_3 (Santoyo et al., 2016; Bhattacharya, 2019; Xie et al., 2020).

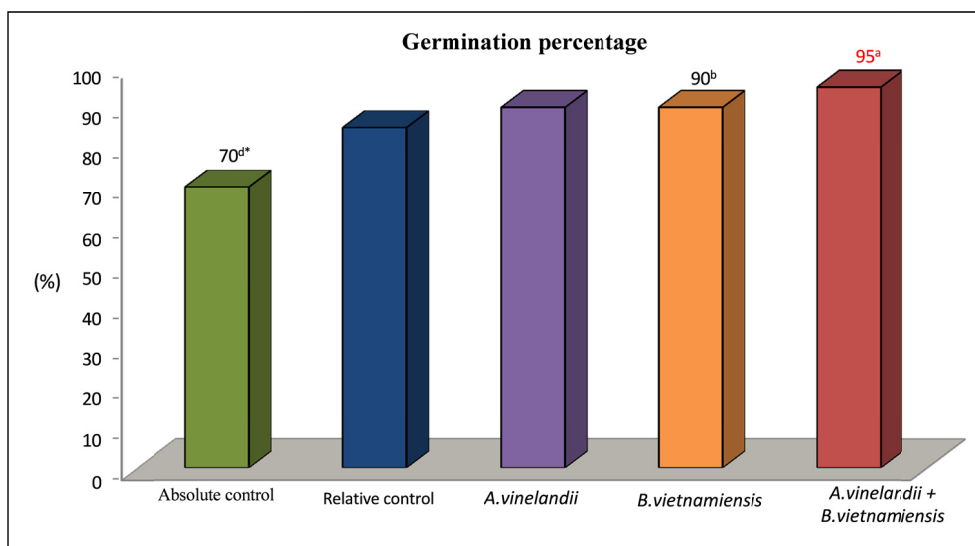


Figure 1. Effect of *Azotobacter vinelandii* and *Burkholderia vietnamiensis* on the percent germination of *Cicer arietinum* and 50% NH_4NO_3

Note. * Values with different letters with statistical difference ($P < 0.05$) according to Turkey.

Figure 2 shows the effect of *A. vinelandii* and/or *B. vietnamiensis* on the phenology: plant height and root length of *C. arietinum* with 50% NH_4NO_3 , where it was evident that individually or in mixture were statistically equal to *C. arietinum* fed with 100% NH_4NO_3 uninoculated. The positive effect of *A. vinelandii* and/or *B. vietnamiensis* supports that when entering the conduction system, both used root metabolites to convert them into phytohormones that accelerated and increased the uptake of NH_4NO_3 at 50% to optimize it to the maximum (Santoyo et al., 2016; Rawat, Sanwal, & Saxena, 2018), compared to *C. arietinum* with 100% NH_4NO_3 uninoculated where it was evident that the uptake of NH_4NO_3 was lower than that observed in *C. arietinum* (Rangjaroen et al., 2015; Saini, Dudeja, Giri, & Kumar, 2015; Schneijderberg et al., 2018). This demonstrates that, individually or in a mixture, *A. vinelandii* and *B. vietnamiensis*, through phytohormonal action from within the roots of *C. arietinum*, optimized the uptake of NH_4NO_3 to 50% (Schneijderberg et al., 2018; Rana et al., 2020a). That prevents the loss of soil fertility, contamination of surface water, as well as the release of N_2O to mitigate global warming (Bhattacharya, 2019; Rai D et al., 2020).

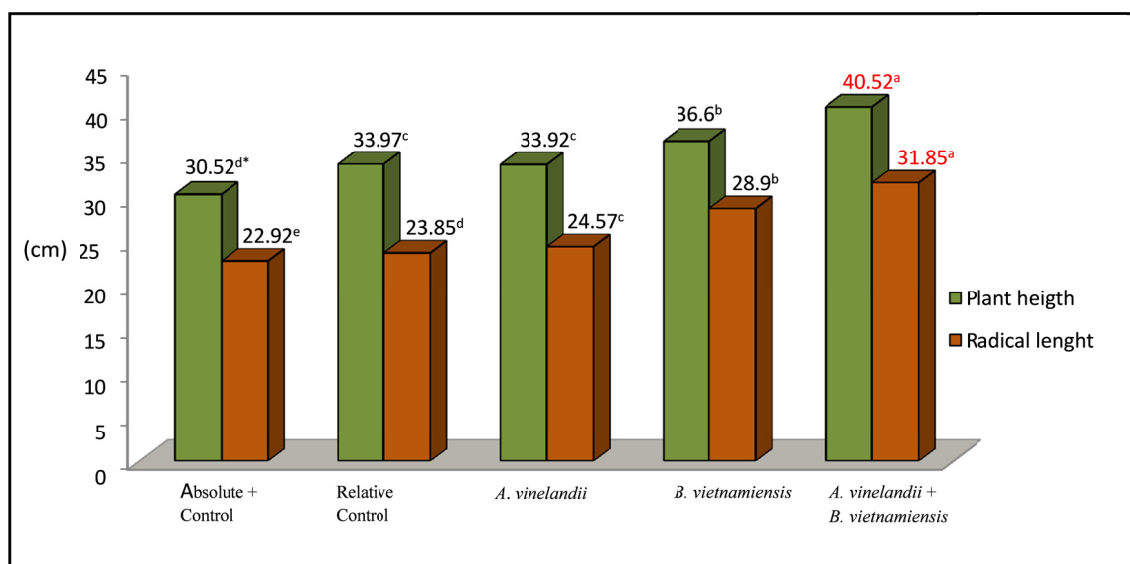


Figure 2. Effect of *Azotobacter vinelandii* and *Burkholderia vietnamiensis* on phenology of *Cicer arietinum* with 50% NH_4NO_3 at seedling stage

Note. *Values with different letters have statistical difference ($P < 0.05$) according to Turkey.

Figure 3 shows the effect of *A. vinelandii* and/or *B. vietnamiensis* on the biomass measured as total fresh and dry weight of the aerial and radical part of *C. arietinum* with NH_4NO_3 at 50% at seedling level, compared to the biomass of *C. arietinum* with NH_4NO_3 at 100% not inoculated, where it was clear that both *A. vinelandii* and/or *B. vietnamiensis* effectively invaded the root conduction system converted compounds of that tissue into phytohormones (Shabanamol et al., 2018; Shahwar et al., 2023), that the uptake of NH_4NO_3 at 50% was optimized so that the values of the total fresh and dry weight inoculated with *A. vinelandii* and/or *B. vietnamiensis* that through a synergistic action of phytohormones effectively optimized the uptake of NH_4NO_3 at 50% (Shahzad et al., 2016; Singh et al., 2017; Wybouw & De Rybel, 2019), thus ensuring the conservation of soil fertility, reducing the possibility of surface water pollution and N_2O generation as a way to mitigate global warming (Saini, Dudeja, Giri, & Kumar, 2015; Segura, Núñez, & Espín, 2020). While the numerical values of *C. arietinum* treated with *A. vinelandii* and/or *B. vietnamiensis* were statistically different from the total fresh and dry weight of *C. arietinum* with 100% of NH_4NO_3 uninoculated where it was evident that excess of NH_4NO_3 (Schneijderberg et al, 2018; Shabanamol et al, 2018).

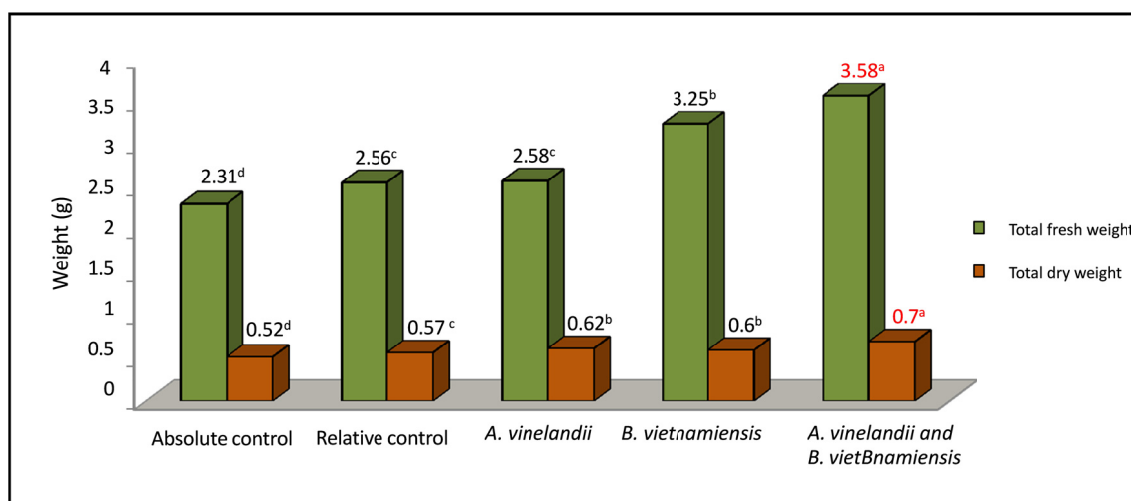


Figure 3. Effect of *Azotobacter vinelandii* and *Burkholderia vietnamiensis* on the weight of *Cicer arietinum* with NH_4NO_3 at 50% at the seedling level

Note. * Values with different letters have statistical difference ($P < 0.05$) according to Turkey.

Figure 4 shows the effect of *A. vinelandii* and/or *B. vietnamiensis* on the phenology of *C. arietinum* at 50% NH_4NO_3 at flowering. It was found that both *A. vinelandii* and *B. vietnamiensis* individually and in mixtures of both caused a positive effect on plant height and root length, by a phytohormonal action by transforming organic compounds derived from root metabolism (Yuan, Liu, & Zhang, 2015; Xie et al., 2020;), to increase plant height and root length. That implies that by having been isolated as endophytes of *L. leucocephala* a forage legume can recognize organic compounds of the root to convert them into phytohormones (Shahzad et al., 2016; Singh et al., 2017; Shahwar et al., 2023), thereby optimizing the uptake of NH_4NO_3 at 50%, that shows that the 100% dose is not what *C. arietinum* requires for healthy growth (Rawat, Sanwal, & Saxena, 2018; Schneijderberg et al., 2018), consequently the inability of the root to such concentration causes loss of fertility (Raiten & Aimone, 2017; Rai et al., 2020), contamination of surface water with the release of N_2O a greenhouse gas that causes global warming (Rana et al., 2020a, 2020b) so both *A. vinelandii* and *B. vietnamiensis* are key to optimize the uptake of NH_4NO_3 at 50%, in that sense the organic matter of the soil is conserved (Yadav et al., 2018a; Shahwar et al., 2023), the contamination of surface water is avoided as well as the generation of N_2O , a way to mitigate global warming (Santoyo et al., 2016; Singh et al., 2017).

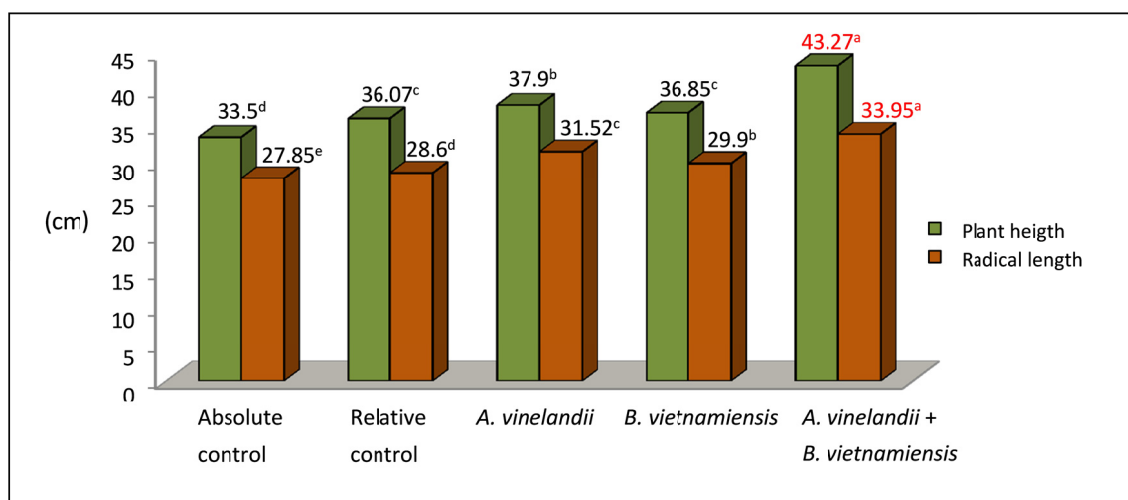


Figure 4. Effect of *Azotobacter vinelandii* and *Burkholderia vietnamiensis* on the phenology of *Cicer arietinum* with NH_4NO_3 at 50%

Note. * Values with different letters with statistical difference ($P < 0.05$) according to Turkey.

Figure 5 shows the effect of *A. vinelandii* and/or *B. vietnamiensis* on the total fresh and dry weight of *C. arietinum* with 50% NH_4NO_3 at flowering. It was evident that both *A. vinelandii* and/or *B. vietnamiensis* individually or co-inoculated reached statistically different numerical values of fresh and dry weight of aero and radical because when invading the root of *C. arietinum* they were able to convert organic compounds from the metabolism of this tissue into phytohormones to optimize the uptake of NH_4NO_3 at 50% (Yadav et al., 2018b; Yan et al., 2018; Wybouw & De Rybel, 2019) in that sense the soil conserves organic matter with the productive capacity, prevents contamination by NO_3^- of surface water and aquifers and also avoids the release of N_2O to mitigate global warming by intensive agriculture (Xing et al., 2016; Yadav et al., 2018a), compared to the same variables response of *C. arietinum* fed with NH_4NO_3 at 100% uninoculated, there it was clear that *C. arietinum* was unable to uptake NH_4NO_3 , due to the absence in the roots of *A. vinelandii* and *B. vietnamiensis* (Singh et al., 2017; Xiao et al., 2017). Consequently, the excess of NH_4NO_3 can cause loss of fertility, contamination of surface water and aquifers as well as the release of N_2O to promote global warming to the detriment of life on planet Earth (Xie et al., 2020; Shahwar et al., 2023).

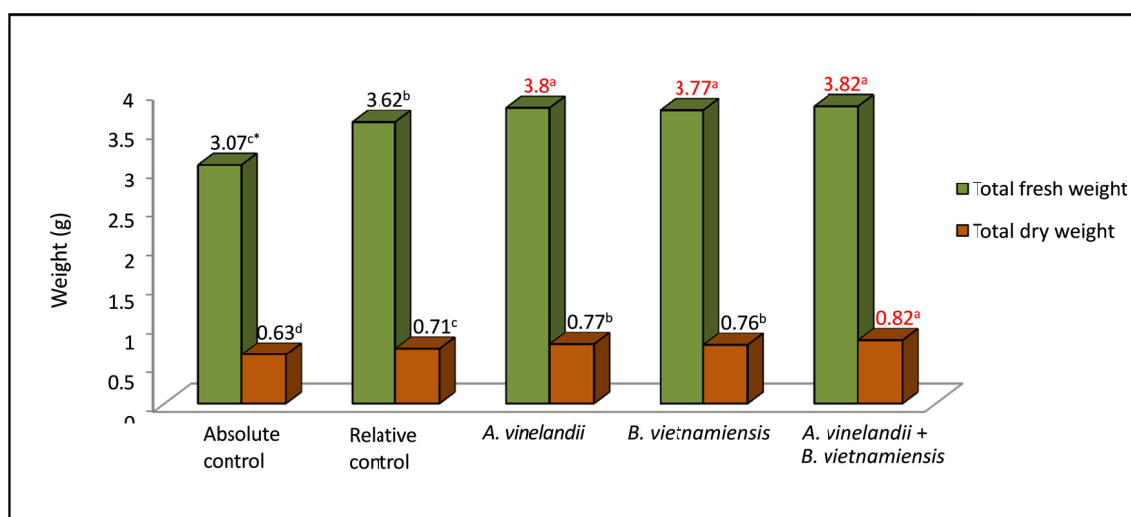


Figure 5. Fresh and dry total weight at flowering stage of *Cicer arietinum* with *Azotobacter vinelandii* and *Burkholderia vietnamiensis* at 50% NH_4NO_3

Note. * Values with different letters have statistical difference ($P < 0.05$) according to Turkey.

Table 1 shows the biochemical profile of *A. vinelandii*, an endophyte of *L. leucocephala*, according to its sugar utilization pattern, characteristic of the *A. vinelandii* species. It also includes biological N_2 fixation by acetylene reduction (data not shown), in addition to the synthesis of n-butanol cysts, a specific differential characteristic of this species (Anjum & Chandra, 2015; Guo et al., 2016; Marentes-Culma et al., 2022). This data is corroborated by the collection strains used to confirm the isolation of *L. leucocephala*. This behavior has been reported in other research (Raiten & Aimone, 2017; Rai et al., 2020;), where *A. vinelandii* has been found to promote plant growth, aiding healthy plant growth in soil poor in mineral N (Rangjaroen et al., 2015; Rana et al., 2021).

Table 1. Biochemical identification of *Azotobacter vinelandii* isolated from *Leucaena leucocephala*

Biochemical test	<i>A. vinelandii</i>
Nitrate reduction	+
Tryptophan	-
Fermentation of glucose	+
Arginine dehydrolase	-
Urease	+
Esculin hydrolysis	+
Gelatinase	+
β -galactosidase	+
Assimilation : D-glucose	+
L-Arabinose	+
D-mannose	+
D-acetyl-D-glucosamine	+
Maltose	+
D-gluconate	+
Capratus	+
Adipate	+
L-malate	+
Citrate	-
Phenylacetate	+
Cyst production	+

Note. * Reaction (+) = positive and Reaction (-) = negative.

Table 2 shows the biochemical profile of *B. vietnamiensis*, are rods Gram negative, whose metabolic behavior of utilization of sugars, fatty acids and tryptamine was similar to a *B. vietnamiensis* strain isolated from *Oryza sativa* (rice), as well as the biological fixation of N_2 (Saini, Dudeja, Giri, & Kumar, 2015; Schneijderberg et al., 2018; Shabanamol et al., 2018) by acetylene reduction (data shown), behavior reported in teosinte isolates in endophytic form (Shahzad et al., 2016; Singh et al., 2017), as well as in this work inside of root of *L. leucocephala* in soil with stress of mineral nitrogen and free phosphates, where this ancestral gramine grows without health problem (Xiao et al., 2017; Xie et al., 2020).

Table 2. Biochemical profile of endophytic *Burkholderia vietnamiensis* from *Leucaena leucocephala*

Biochemical test	<i>Burkholderia vietnamiensis</i>
Nitrate reduction	+
Tryptophan	+
Fermentation of glucose	-
Arginine dehydrolase	+
Urease	+
Esculin hydrolysis	+
Gelatinase	+
β -galactosidase	+
Asimilation :	
D-glucose	+
L'arabinose	+
D-mannose	+
D-mannitol	+
N-acetyl-D-glucosamine	+
Maltose	-
D-gluconate	+
Capratus	+
Adipate	+
L-malate	+
Citrate	+
Phenylactate	+

Note. * Reaction (+) = positive and Reaction (-) = negative.

In Figure 6 when staining with Lugol iodine, the round, double-walled dormancy structure called a cyst was observed. In the literature it is reported that *A. vinelandii* does not produce endospores, but it does produce cysts, structural and physiological modifications of its vegetative cells with resistance to: desiccation, mechanical disintegration, ionizing radiation, ultraviolet and predation, but not with resistance to heat (Chowdhury-Paul et al., 2018; Martínez-Ortiz et al., 2020; Rana et al., 2020a), cysts are not completely inactive compared to bacterial endospores of the genus *Bacillus*, the production of cysts is the main property that differentiates *A. vinelandii* from other genera of N_2 -fixing, free-living or symbiotic endophytic plant growth promoting bacteria (Romero et al., 2016; Xing et al., 2016; Segura, Núñez, & Espín, 2020).

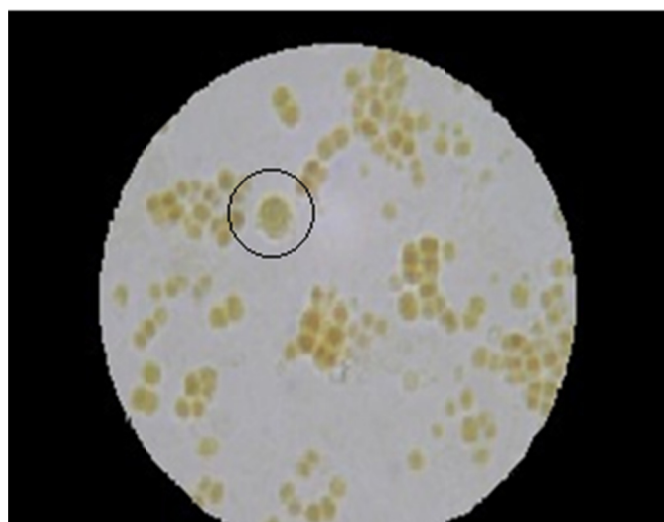


Figure 6. Cysts of *Azotobacter vinelandii* from *Leucaena leucocephala* under microscopic observation at 100 $\times$, cysts (arrows) on Burk agar with 1% Butanol for 1 week/30 °C

4. Conclusion

It is evident that *Leucaena leucocephala* a wild legume recommended as green manure and livestock fodder has more useful properties for sustainable agriculture. It is a source of genera and species as well as *A. vinelandii* and *B. vietnamiensis* of plant growth-promoting endophytic bacteria compatible with *C. arietinum* for the optimization of NH_4NO_3 at 50% of recommended dose. This allows the application of this fertilizer to be regulated, but not thereby compromising the healthy growth of this legume with high nutritional value for humans and animals. This does not involve surface water contamination or the release of N_2O , which prevents global warming, which is detrimental to the quality of life on Earth during agricultural production of *C. arietinum*.

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Experimentation IYLA, JLIC and JMSY conceptualization JMSY and RERN, methodology, IYLA, JLIC, EMGBP, RERN, MEA and JMSY software, JLIC and JMSY; validation, IYLA, RGG and JMSY; results YILA and JMSY.; investigation, IYLA, EMGBP, RGG, MEA and JMSY; resources, JMSY writing—original draft preparation JMSY writing—review and editing, JMSY; visualization, JLIC, RERN and JMSY; supervision JMSY. All authors have read and agreed to the published version of the manuscript.

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