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In vitro rhizobia response and symbiosis process under Aluminum stress

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Running Title: Al-stress on rhizobia and symbiosis

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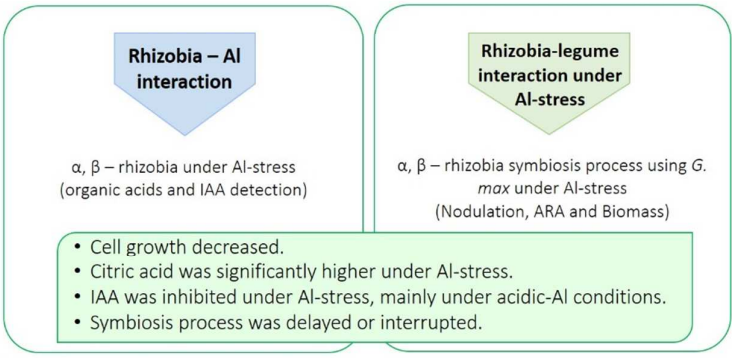
Graphical Abstract



ABSTRACT

Aluminum (Al) toxicity is a major problem affecting soil fertility, microbial diversity, and nutrients uptake of plants. Rhizobia response and legume-interaction under Al conditions are still unknown and it is important to understand how to develop and improve legume cultivation under Al-stress. In this study, rhizobia-response was recorded under different Al-concentrations. Al-effect on rhizobial cells was characterized by combination with different two pH conditions. Symbiosis process was compared between *α*- and *β-rhizobia* inoculated onto soybean varieties. Rhizobial cell numbers was decreased as Al concentration increased. However, induced Al-tolerance considerably depended on rhizobia types and their origins. Accordingly, organic acid results were in correlation with growth rate and cell density which suggested that citric acid might be a positive selective force for Al-tolerance and plant-interaction on rhizobia. Al-toxicity delayed and interrupted the plant-rhizobia interaction and the effect was more pronounced under acidic conditions. *Burkholderia fungorum* VTr35 significantly improved plant growth under acid-Al stress in combination with all soybean varieties. Moreover, plant genotype was an important factor to establish an effective nodulation and nitrogen fixation under Al-stress. Additionally, tolerant-rhizobia could be applied as an inoculant on stressful agroecosystems. Furthermore, metabolic pathways have still been unknown under Al-stress.

Key words: Aluminum-stress, *Bradyrhizobium*, *Burkholderia*, citric acid, *Glycine max*

INTRODUCTION

Aluminum (Al^{3+}) is one of the most common metals in the earth. Al-toxicity limits plant production worldwide, particularly in acidic soils. In Americas, 70% of tropical soils experience limitations related to acidic soil, Al- and Mn-toxicity, in addition to the deficiency of other elements such as Ca, Mg, P (Martinez-Viera et al. 2006). Thus, there are many types of plants that are affected by different Al ion concentrations in soils. Furthermore, the combination of Al and low pH disrupt many pathways and physiological functions in plants such as root elongation, root hair formation and nutrients uptake (e.g., K, Ca, N,) (Girma et al. 1997, Nian et al. 2003).

The main symptom of Al-toxicity is inhibition of root growth in the plant. Moreover, Al can disrupt many other functions including root hair elongation and nutrient uptake (especially Ca and K). Al can induce oxidative stress, disrupt cytoskeleton and apoplastic processes, and affect intracellular transport (Kochian et al. 2005). Acidic soils now encompass 35% of arable land and therefore, Al-toxicity is a major selective pressure for plant adaptations. Many species have evolved several mechanisms to improve their survival on acidic soils. Even before these mechanisms were fully understood, mechanisms were divided into those that were likely to exclude Al from the root (i.e., exclusion or resistance mechanisms) and those that would enable plants to safely accommodate Al once it enters the symplast (i.e., tolerance mechanisms) (Khan et al. 2009). In previous studies, tolerance mechanisms were distinguished from resistance mechanisms. Exclusion mechanisms were predicted to depend on transport systems that export Al from the symplast or exudate ligands that bind Al and limit its uptake into the cytosol (Khan et al. 2009: Kochian et al. 2005).

In bacteria, some non-rhizobia are commonly recognized to be Al-resistant at low pH, and some studies indicate that rhizobia could also be tolerant bacteria (Vargas and Graham

1988, Wood et al. 1988). Regarding the toxicity of Al, Johnson and Wood (1990) reported that Al was taken up and bound to DNA of both sensitive and tolerant bacterial strains but DNA synthesis was not affected by Al in tolerant strains. However, Al-toxicity mainly depends on pH during the bacteria-plant interaction. Additionally, Richardson et al. (1988) found that Al depressed *nod* gene expression at low pH. Bioavailability of Al in soils remains low at neutral pH due to the adsorption of minerals in association with organic matter or acids and the insolubility of formed hydroxide complexes. In acidic soils where heavy-metal activity is relevant, the presence of available Al inhibits nodulation (Bååth et al. 1998). In recent years, heavy metals are the most important inorganic pollutant such as Cu, Ni, Cd, Zn, Cr, Pb. Furthermore, the presence of Al has been recognized as a serious pollution problem for soil and water acidification because Al can be released (solubilized) from its natural reservoirs under acid rain (Graham 1992; Soares et al. 2014; Mendoza-Soto et al. 2015). Thus, the nature and site of Al ion actions remain unknown and undefined in rhizobial cells, especially its effects on the symbiotic process (Piña and Cervantes 1996). In our study, the response of the rhizobia under Al-stress was recorded. The objective of this study is to investigate and characterize the effect of Al on α-rhizobia and β-rhizobia, including the symbiosis process under combination with two different pH conditions, where responses of Al-tolerant and Al-sensitive strains and soybeans were compared. In this way, characterization of plant growth promoting rhizobia, nitrogen fixation, and nodulation were evaluated.

MATERIALS AND METHODS

Strains and plant sample

Four strains including two types of α-rhizobia (*Bradyrhizobium diazoefficiens* USDA 110 and *Rhizobium pusense* VAF12.43 (LC107564)) and two types of β-rhizobia (*Burkholderia fungorum* VTr35 (LC104296.1) and *Burkholderia symbiont* VG10B (LC107593)) were

selected in the present study. *B. fungorum* VTr35, *B. symbiont* VG10B, and *R. pusense* VAF12.43 were isolated from different agro-ecological conditions in South America including the Andes, savanna and valley areas in Venezuela (Artigas 2015). VTr35 was reported as an acidic-Al tolerant strain (Artigas 2015). The strain *B. diazoefficiens* USDA 110 was referred to sensitive strain to high Al ion concentration. Plant samples in the present study were *Glycine max* 'INIA' (domesticated in tropical conditions by INIA-Venezuela) and *G. max* 'Enrei' (traditional Japanese seed).

Stress-tolerance screening

The strains were evaluated for growth under high concentration of Al at different pH. Al-tolerance of isolates was tested by recording their ability to grow under the following concentrations of $AlCl_3$ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and pH conditions: 0 (as control) 0.1, 0.5, 1 and 2 mM, at acidic (pH 4.5) and neutral pH (pH 6.8) conditions. The isolates were first grown in Yeast Mannitol (YM) broth for 4 days at 28 °C and then 5 µL of cell suspensions at 10^9 cells mL^{-1} were transferred to YM Agar (YMA) plates and YM broth under stress conditions at 28 °C for 4 to 10 days. Growth of strains in YMA was estimated in comparison to the control treatment as follows: –, no growth; +, weak growth (10–20% in relation to the control); ++, good growth (30–60% in relation to the control); and +++, very good growth (similar to or same as the control) (Somasegaran and Hoben 1994). The colony-forming unit (CFU) was calculated by $O.D_{600nm}$ after 5 days in YM broth under Al-stress conditions. These experiments were carried out in triplicate for each isolate.

Organic acids analysis

The isolates were grown in YM broth for 4–5 days at 28°C and their concentrations were adjusted to 10^9 cells mL^{-1} as described by Vincent (1970). Subsequently, these rhizobial cells

were inoculated into YM broth under Al-stress conditions. After incubation, the culture was centrifuged at 5000 rpm for 10 min. The supernatant was filtered with 0.22 µm sterile filter unit (Millex®-GV, MILLIPORE, Cork, Ireland). Subsequently, the extracted solution was analyzed for organic acid by Shimadzu DGU-20A high-performance liquid chromatograph (HPLC) equipped with a Shim-pack column SCR-101N (Shimadzu Corporation, Kyoto, Japan). For several acids such as citric, fumaric, tartaric, succinic and malic were used in 10 mM to 1 M concentrations. The retention time was 25 µL 90 min^{-1}. The analysis was performed by GC-Solutions Software (Shimadzu Corporation, Kyoto, Japan).

Indole-3-acetic acid (IAA) analysis

Each strain was inoculated into YM broth containing 100 mg L^{-1} L-Tryptophan and incubated at 28 °C for 5 days in the dark to quantify the production of indole-3-acetic acid (IAA). Then, the cell suspensions were centrifuged at 10.000 rpm for 15 min to remove cells. IAA concentration in the supernatant was determined by the Salkovski colorimetric technique (Glickmann and Dessaux 1995) by measuring absorbance at 530 nm using a spectrophotometer (Ultrospec 3300 pro, Amersham Biosciences, Cambridge, United Kingdom). These experiments were carried out in triplicate for each strain included under different Al-stress conditions.

Plant tests for symbiosis analysis under Al-stress conditions

The plant test was performed *in vitro* conditions. A free-hormone medium was used as described by Norris and Date (1976) and this media was prepared for symbiosis in combination with aluminum. The medium was autoclaved 120 °C / 0.2 MPa for 15 min. Al-stock ($AlCl_3.6H_2O$, 1M) was filtered and added to the medium at 40 °C. There were two pH conditions, namely, acidic (pH 4.5) and neutral pH (pH 6.8) in combination with different

concentrations of Al ($AlCl_3$) which are 0 (as control), 0.1 (minimum tolerant), 0.5, 1, and 2 mM.

Strains of *B. fungorum* VTr35, *B. symbiont* VG10B, *B. diazoefficiens* USDA 110 and *R. pusense* VAF1243 were grown in YM broth for 4–5 days at 28 °C to obtain 10^9 cells mL^{-1} as described by Vincent (1970). Prior to the inoculation, seeds were surface-sterilized with 70% ethanol for 30 s, 3% (v/v) of sodium hypochlorite for 2 min and then washed 4 times with sterile distilled water. Subsequently, these rhizobial cells were inoculated into *G. max* 'INIA' and 'Enrei'. Plants were grown in an incubator (Fli 2000 - EYELA, Tokyo Rikakikai Corporation, Tokyo, Japan) under axenic and sterile conditions. A sterilized N-free nutrient solution (Sylvester-Bradley et al. 1983) was added to the tube until 60% moisture and this level was maintained throughout the growth period. Plants were cultivated for 30 days in the growth chamber under a 16 h light / 8 h dark photoperiod at 28 °C. The observations of plant-rhizobia interaction were at 5^{th}, 15^{th}, 20^{th} and 30^{th} days. However, the harvest was done twice at 15^{th} and 30^{th} days. For all the treatments, five replicates per each strain were performed. One replication contained one plant per tube (25 mm × 200 mm, Pyrex) with 50 mL of medium. Non-inoculated plants served as control treatments (Vincent 1970).

Nitrogen acetylene reduction activity

Plant intact with the root nodules were collected after 15 and 30 days of culture for nitrogen fixation assessment using acetylene reduction assay (ARA). ARA was measured by a Shimadzu GC-2014 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a Porapak N column (Agilent Technologies, Santa Clara, USA) with 30 min incubation, followed by determination of root nodule numbers. Plant weights were measured after they were dried at 80 °C for 48 h.

Statistical analyses

Statistical analyses were made by Tukey's test using the Statistica software v.12.0 (StatSoft, Tulsa, USA). A value of $p \leq 0.05$ was considered as an indication of statistical significance.

RESULTS

Effect of Al on cell viability and organic acid production of rhizobia cells

Growth density of rhizobial cells generally decreased as Al ion concentration increased in both acidic and neutral pH conditions (Table 1 and Fig. 1). However, Al-toxicity on cell growth and colony density was more pronounced in a combination of Al and acidic pH than those in a combination of Al and pH 6.8. In α-rhizobia, *Bradyrhizobium diazoefficiens* USDA 110 and *Rhizobium pusense* VAF1243 in acidic condition (pH 4.5), the results were much lower than those a neutral condition (pH 6.8) without Al (Table 1, Fig. 1A-B). In contrast, β-rhizobia cells were slightly or moderately affected by Al under neutral pH. In acidic condition, the colony density and cell growth of *Burkholderia symbiont* VG10B and *Burkholderia fungorum* VTr35 were similar to those in neutral condition with 0 mM Al (Table 1, Fig. 1C-D). As Al concentration increased to 0.5 mM, a sharp decline was observed in colony density of β-rhizobia (Fig. 1C-D). However, the response of rhizobial cells under Al-effect showed some differences which indicate a form of resistance in rhizobial cells.

Accordingly, organic acids including malic, citric, tartaric, succinic and fumaric acid produced by these cells were analyzed under Al-stress conditions as shown in Table 2. The concentration mainly differs based on the type of rhizobia and stress condition. There are no significant differences between the types of rhizobia under Al-stress with tartaric acid. Succinic acid concentration varied according to the type of rhizobia, particularly for α-rhizobia at both pH conditions. Malic and citric acid concentrations were relatively high

among these organic acids (Table 2). The concentrations of malic acid produced by different rhizobia were correlated to the cell growth rates. However, the concentration of malic acid produced by different rhizobia showed a decreasing tendency as Al concentration increased in either neutral or acidic pH (Table 2). The citric acid concentration was higher in *B. diazoefficiens* USDA 110 and *B. fungorum* VTr3 under Al-stress at neutral pH conditions than those in *R. pusense* VAF1243 and *B. symbiont* VG10B (Table 2). No significant correlations were found between the changes of citric acid concentration at neutral or acid pH under Al-stress. Additionally, the concentration of citric acid was significantly higher compared to other organic acids (Table 2). Our results proposed that citric acid was essential for survival and activities of rhizobial strains. Thus, a correlation between Al-toxicity and citric acid was evidenced, especially under neutral pH and high Al concentration.

IAA production under Al-stress conditions

The IAA concentration for each rhizobial strains was measured under Al-stress conditions at both acidic and neutral pH (Table 2). The IAA concentration decreased in the neutral condition whereas the Al concentration increased in all rhizobial strains. The IAA concentration was undetectable when it was higher than 0.1 and 0.5 mM under Al-stress in α-rhizobia and β-rhizobia, respectively. In addition, the IAA production was completely inhibited under acidic Al-stress conditions. Al-toxicity was observed at neutral pH when the IAA was no detected under high Al concentration.

Plant – rhizobia interaction under Al-stress conditions

During the *in vitro* cultivation, plants were analyzed for symbiosis responses in two types of rhizobial cells (α- and β-rhizobia) and two types of seed varieties. After 5 days, nodules had not yet been developed. However, nodule differentiation was observed 10 days after

inoculation, especially in control (0 mM) and 0.1 mM under neutral pH. The number of detached nodules after 15 and 30 days was calculated (Fig. 2). Both types of rhizobial strains showed less than 10 nodules per plant (depending on rhizobia and soybean variety) (Fig. 2A-2B), and nodulation occurred until stage 2 in low proportion. After 15 days, the number of nodules decreased until no nodulation occurred, whereas Al concentration increased in either neutral or acidic conditions in *G. max* 'INIA' and 'Enrei'. The rhizobial response under low-Al concentration (lower than 1 mM) in both pH conditions were significantly higher with *B. fungorum* VTr35 compared to the other strains, although the relative low rhizobial response was also observed in *B. diazoefficiens* USDA 110 (Fig. 2A-2B). Nevertheless, there was a lower number of nodules in *G. max* 'Enrei' than in *G. max* 'INIA' when the nodules were formed. Furthermore, the total amount of nodules did not include nodules at first stage. However, some nodules at first stage were observed under 0.5 and 1 mM Al with *B. fungorum* VTr35 and *B. diazoefficiens* USDA 110, mainly at 6.8 pH, during the harvest time.

After 20 days, there was no synchronization on the nodulation process due to the presence of nodules at the first and third stage (completely developed) at the same time under Al-stress. However, the nodules were completely developed during the final observation until harvest time (30 days). Under neutral conditions, Al-effect was more evidenced (Fig. 2C-2D. Similarly, Al-toxicity was more pronounced under acid pH after 15 days (Fig. 2C-D). Moreover, no plant-rhizobia interaction or effective nodules was established under high Al concentration for rhizobia including *R. Pusense* VAF1243 and *B. symbiont* VG10B. In *B. diazoefficiens* USDA 110 some ineffective nodule was developed per plant at 2 mM (Fig. 2C). Furthermore, three rhizobia-interaction with 'Enrei' were strongly interrupted, which appears to be non-tolerant to Al ion concentration (Fig. 2D). In addition, *B. fungorum* VTr35 nodulation activity was confirmed under high Al concentration in both cultivars and under both pH conditions. The leaf size or coloration changed under Al-stress due to the inhibition

of nitrogen fixation (Fig. 3A). Roots, as well as root hairs or laterals roots were affected by the presence of Al (Fig. 3B). Interestingly, morphological observation of the nodules could differentiate the leghemoglobin reduction until very low levels (*B. fungorum* VTr35) or even in non-leghemoglobin appearance (inactive nodule, *B. diazoefficiens* USDA 110) (Fig. 3C). In β-rhizobia, some dark coloration was observed in the external appearance of nodules.

Nodulation leads to nitrogen fixation activity which increases the plant biomass (in most of the cases). Thus, nitrogen fixation activity (Fig. 4) and biomass (Fig. 5) were verified at 15th days. Consistent with nodulation, ARA concentration decreased as Al concentration increased. The highest ARA concentration was found in plants inoculated with *B. fungorum* VTr35 (Fig. 4A and 4B). However, ARA was not detected in 'Enrei' under acidic pH, although ARA was lower in 'Enrei' with *B. fungorum* VTr35 under pH 6.8 (Fig. 4B). Regarding the length (data not showed) and biomass, there was no significant difference between α-rhizobia and β-rhizobia (Fig. 5). However, the biomass was slightly increased with *B. fungorum* VTr35 inoculated on *G. max* 'INIA' and 'Enrei' at 1 mM or 2 mM Al conditions (Fig. 5).

The length, dry weight (DW) and ARA concentration were recorded after 30 days using as host trap soybean 'INIA' and 'Enrei', respectively (Tables 3 and 4). The length, DW of roots and shoots and ARA concentration decreased, whereas Al concentration increased in all groups. However, the rhizobia improved the stress response in both types of seeds compared to the control (non-inoculation). Furthermore, the plant-interaction from two different rhizobia (*R. pusense* VAF1243 and *B. symbiont* GV10B) was very similar and strongly affected by Al, especially from 1 mM and independent of the soybean variety (Tables 3 and 4). However, the length and DW of roots and shoots were similar with *B. fungorum* VTr35 in combination with 'INIA' across different Al-stress conditions (Table 4). Nitrogen fixation results were similar to those at 15 days. Interestingly, a combination of *B. diazoefficiens* USDA 110 at neutral pH showed a high ratio of fixed nitrogen under Al-stress conditions. However, *B. fungorum*

303 VTr35 displayed significant results in ARA concentration under a combination of Al-acidic
304 conditions.
305
306 **DISCUSSION**
307 In this study, α- and β-rhizobia strains were analyzed for their abilities to tolerate different
308 Al concentrations under acidic or neutral pH conditions. Adverse effects on the growth of
309 rhizobia and plant-rhizobia interaction increased as Al concentration increased in both pH
310 conditions. The combination of increased Al concentration and acidic pH had a greater Al-
311 toxicity on rhizobia growth and bioactivity. Similarly, different resistance abilities of the four
312 types of rhizobia to the Al-stress conditions were also observed in the present study. These
313 results suggest that some organic acids such as citric acid are involved in the pathway of Al-
314 tolerance and the symbiosis process under Al-stress conditions differs depending on the Al
315 concentration, pH, type of rhizobia (including origin) and plant variety (cultivation history).
316 Effects of heavy metals on rhizobial composition within soils, nodule environments or
317 different legume genotypes have been contradictory (Wani et al. 2007, 2008). Hirsch et al.
318 (1993) demonstrated that *R. leguminosarum bv. trifolii* was altered by long-term exposure to
319 heavy metals, which caused an ineffective nodulation. When 50–200 mg kg^{-1} soil of Co, Cu,
320 Cd and Zn was added deliberately to soils used for *Lablab purpureus* cultivation, these metals
321 significantly decreased growth, nodulation and nitrogenase activity of plants in both pot and
322 field experiments (Younis 2007). In contrast, Angle and Chaney (1991) described that the
323 symbiotic association between *Medicago sativa* and *R. meliloti* was not affected by high metal
324 contamination. Furthermore, Ferreira et al. 2012 found that *Rhizobium* strains isolated from
325 acidic soil were highly tolerant to Al. Similarly, *Burkholderia* strains (UFLA3-154) and
326 *Bradyrhizobium* strains (UFLA 03-84) as nodulating bacteria have high Al ion tolerance
327 because were isolated from acidic areas (Soares et al. 2014). Other studies have also reported

that rhizobia strains and non-rhizobia were resistant to Al under low pH condition (Vargas and Graham 1988; Wood et al. 1988). In acidic conditions where heavy-metals activity is relevant, Al-availability inhibits nodulation (Bååth et al. 1998). Our present results were consisted with previous data which showed inhibitory effects on rhizobia cell growth under high Al concentration with and without acidity. Regarding Al-effect, Johnson and Wood (1990) found that Al was taken up and bound to DNA of both sensitive and tolerant bacterial strains, but DNA synthesis in tolerant strains was not affected. Several authors have reported that Al has damaged or depressed specific genes on rhizobia (Richardson et al. 1998, Johnson and Wood 1990). Furthermore, response variability of the four strains to Al-stress was recorded in our study. These results indicated a variation in Al-stress response depending on the type of rhizobia. In more detail, Al-response could have changed according to strain origin which could lead to microbial populations with higher tolerance to metals (Bååth et al. 1998).

It is important to mention that Al-tolerance and successful symbiosis are influenced by the presence of several compounds for example organic acid, phytohormones and flavonoids. under stress conditions. Regardless of the plant, production of organic acids by rhizobial cells (such as citric and malic acid) may play an important role in cell survival and symbiosis process under some stress. In our case, citric acid could be one of the explanations for survival and effective symbiosis in resistant-rhizobial strains. *B. diazoefficiens* USDA 110 and *B. fungorum* VTr35 released a high amount of citric acid under Al-stress, which might act in microbial chemotaxis and metal detoxification. Citric acid indicated the highest binding activity for Al followed by malate and succinate (Panda et al. 2009). Moreover, concentrations of malic acid produced by rhizobial cells changed depending on Al-stress which indicates it may serve as a marker for the detection of Al-stress in the four types of rhizobia used in this study. Additionally, succinic acid has been reported as an important compound in the metabolic pathway of α-rhizobia species especially in *Bradyrhizobium* (Lyer et al. 2016).

353 Succinic acid might mediate the repression of sugar utilization in rhizobia which is reflected in
354 a high concentration of succinic acid released by rhizobia under Al-stress conditions.
355 Moreover, it is well known that the capacity of organic acids to increase P availability not only
356 results from plant rhizosphere but also from their capacity to form stable complexes with some
357 metals such as Al and Fe (Lyer et al. 2016; Nail et al. 2003; Paredes-Mendoza and Espinosa-
358 Victoria 2010). In addition, organic acids could increase the availability of other soil
359 micronutrients such as Mn, Al, and Zn when pH decreases in the rhizosphere or by chelation
360 of micronutrients (Paredes-Mendoza and Espinosa-Victoria 2010). It is important to mention
361 that some organic acids were detected depending on the type of rhizobia. However, further test
362 and analysis are necessary for these organic acids obtained in our results. In fact, Al-
363 bioavailability and toxicity are greater in soils under Al-acid conditions. Physical-chemistry
364 reactions amended with limited minerals, their up-taking and the mineral-adsorption remains
365 low due to the association between organic matter and insolubility of formed hydroxide
366 complexes (Bååth et al. 1998). Although, metabolic pathways are still unknown under heavy
367 metal stress conditions. However, there is a great possibility for these organic acids to promote
368 neutralization of Al ions in the environment and help root cells to stimulate interactions.

369 It is necessary to understand the ecosystem, including soil microorganisms to secure a
370 successful development of plants. Soil microorganisms are very sensitive to moderate-heavy
371 metal concentrations (Giller et al. 1998). High levels of heavy metals in soil or water solutions
372 can cause dramatic changes in microbial compositions and activities (Bååth et al. 1998;
373 Lakzian et al. 2002; Paudyal et al. 2007). Multiple genes encoding for heavy metal and
374 antibiotic resistance are frequently found on the same plasmids and/or transposons and confer
375 co-resistance (Summers 2002). In most soils, Al-toxicity could induce restrictions in root
376 growth by increasing ionic strengths of soil solutions (Bruce et al. 1988). Considering that

sensitive and tolerant strains have the same potential genes to tolerate acidic conditions, an unknown mechanism might exist in tolerant strains to repair DNA damage caused by Al.

Furthermore, Arora et al. (2010) reported that species such as *Sinorhizobium meliloti* and *Bradyrhizobium* growing *ex-planta* were extremely sensitive to Al because Al can affect enzymatic activities. This finding is consistent with our results for α-rhizobia. Besides, *Burkholderia* strains can survive under heavy metal toxicity using the protective effect of siderophores (Mathew et al. 2016). Protection potential against heavy metal in pyochelin and ornibactin may stem from their capacities to bind other metal irons. This protective effect suggests that improved growth may allow an increased resistance to heavy metals but do not enhance iron assimilation in the presence of siderophores (Mathew et al. 2016). In β-rhizobia, this finding is consistent with the growth or cell abilities to tolerate Al. However, those β-rhizobia were sensitive to high Al-concentration during plant-interaction as α-rhizobia. These results implied that Al-tolerance ability for different rhizobia not only depended on cell survival but also plant-rhizobia interaction.

Al-effect on the symbiotic process was characterized in different species of rhizobia which established an ineffective or effective symbiosis with two varieties of soybean. *G. max* 'Enrei' is frequently used in Japan and worldwide. 'Enrei' is successful in the interaction with *B. diazoefficiens* USDA 110 and also express high biomass performance (Shimomura et al. 2015). In our study, it was used as sensitive soybean variety for plant-interaction under acidic-Al-stress conditions. Accordingly, our results demonstrated that this combination of plant-rhizobia was strongly affected by Al. The other variety, *G. max* cv. 'INIA' was domesticated in tropical conditions in Venezuela where Al-interaction and acid-Al interactions have been demonstrated in savanna ecosystem and in other areas of Venezuela, respectively. Based on cultivar performances in terms of growth under different stress conditions, the sensitivity of

401 seed and bacteria led to physiological disorders in crops. Our findings were consistent with
402 Indrasumunar et al. (2012) for soybean cultivation in acidic soils and it is necessary to apply
403 acid-tolerant rhizobia which should be isolated from soybean or legume root nodule grown in
404 acidic soils.

405 Accumulation of Al in the cap, epidermis and outer cortex of the root in soybean cultivars
406 are possible and these root tissue can strongly bind to Al (Nian et al. 2003). The plants might
407 supply nutrients or increase the nutrient uptake at 0.1 mM Al due to the changes in membrane
408 permeability and the formation of the protonated complex on root surfaces (Perez-Galdona
409 and Kahn 1994; Girma et al. 1997). Furthermore, the plant-rhizobia interaction was very
410 sensitive to high Al-concentration, especially in sensitive strains (Bååth et al. 1998; Wani et al.
411 2008). Wood and Cooper (1988) found that the proliferation was inhibited in *Lotus*-rhizobia at
412 50 μM Al concentration. Significant pH changes might affect the relative concentration of
413 various Al species and their ligands and therefore result in Al-toxicity (Richardson et al. 1988).
414 Furthermore, pH changes in combination with Al were detrimental to root nodule bacteria,
415 growth rates and lower final rhizobial cell densities. Our findings also suggested that there was
416 a limit for the Al-accumulation in the cell might be between 0.5 and 1 mM due to the
417 symbiosis process at this concentration. Moreover, high Al ion concentration inhibited the
418 rhizobia-plant interaction, especially in α-rhizobia or sensitive strains obtained from other type
419 of rhizobia. In fact, rhizobia-legume molecular signaling exchange was interrupted and
420 subsequently, nodulation and nitrogen fixation were in detriment or completely interrupted.
421 The concentration of Al may reduce isoflavonoid exudate and inhibit induction of *nod* genes
422 (Mendoza-Soto et al. 2015). In this study, the production of IAA as the main auxin was
423 measured, which can explain why plant length and biomass increased under some Al-stress
424 conditions. However, IAA production was completely inhibited under certain stress conditions
425 using the colorimetric method. Rhizobia produced IAA at 0 mM Al and the concentration

differed depending on the type of rhizobia and their ability to use isomer of L-tryptophan that was consistent with results reported by Frankenberger and Muhammad (1995). These studies demonstrated that various rhizobia produced a larger amount of IAA when used with D-tryptophan compared to L-tryptophan. However, it is common to find rhizobia that used as resource L-tryptophan in culture media. In addition, IAA or other auxin production could be affected by the external presence of trace metals and vitamins, and these compounds had variable effects on IAA production (Frankenberger and Muhammad 1995). Our findings showed that Al had an inhibitory effect in basic culture media. In the other side, there were different compounds including Ca^{2+} and $MnSO_4$ during the plant culture which could promote bio-production of IAA or an intermediate-product of auxin during the establishment of symbiosis (Datta and Basu 2000, Frankenberger and Muhammad 1995). Some studies found that IAA-deficient mutant of *B. elkanii* or *B. japonicum* significantly reduced the nodule number on soybean 'Enrei' roots which was restored by exogenous application of IAA (Fukuhara et al. 1994). The results indicated that the nodulation in this cultivar was mainly controlled by IAA (Fukuhara et al.1994). This finding may be one of the reasons why our results demonstrated low nodulation in this cultivar, which was independent of the rhizobia type (Frankenberger and Muhammad 1995).

Accumulation of heavy metals (such as Al, Zn, Cu, Ni, Cr, Cd, Hg and Pb) causes variations in different ecological systems. Some of them not only have a toxic effect on living organisms, but also, they are irreversibly immobilized in soils. These changes in the ecosystem can lead to losses in soil fertility (Hairiah et al. 1995). However, there is limited information about the effect of heavy metals on molecular signals between rhizobia and their host legumes. Nodule morphology was characterized by the presence of leghemoglobin and these nodules obtained from the interaction of α-, β-rhizobia with 2 types of soybean varieties. Soybean rhizobia obtained from stressed locations were expected to establish a successful

451 symbiosis under stress compared to those from sensitive soybeans. Plant biomass was severely
452 inhibited under an acidic condition which was consistent with previous studies and suggested
453 that nodulated legumes were more sensitive to Al and Mn toxicity than plants fertilized with
454 N-fertilizer (Hungria and Vargas 2000). *Mesorhizobium loti* is Al-tolerant but *R.*
455 *leguminosarum bv. trifolii* can be affected by Al through reducing *nodA* gene expression and
456 affecting biological nitrogen fixation (Johnson and Wood 1990). In addition, Richardson et al.
457 (1988) found that 7.5 mM Al depressed *nod* gene expression at low pH (4.8). These results are
458 consistent with our study which showed that nodulation was delayed, and the number of
459 nodules was reduced in high Al-concentration, especially in sensitive strains such as *R.*
460 *pusense* and *B. symbiont*. Furthermore, under Al-stress, soybean nodules showed a declining
461 nitrogenase activity, whereas Al was accumulated in their infected zone which indicates the
462 presence of high Al in all symbionts. Bacteria under excessive Al utilize Fe transport systems
463 for Al uptake which interfere with their ability to capture Fe that is an essential micronutrient
464 required for rhizobial nitrogenase activity (Mendoza-Soto et al. 2015; Mathew et al. 2016).
465 Arora et al. (2010) indicated that *S. meliloti* RMP5 and *Bradyrhizobium* BMP1 were
466 significantly depressed under Al-stress mainly due to various enzymatic activities for nitrate
467 and nitrite reduction, nitrogenase and uptake of hydrogenase (Mendoza-Soto et al. 2015).

468 In conclusion, the degree of toxicity induced by different Al concentrations varies
469 considerably depending on plant species or genotype among other factors (Blair et al. 2009).
470 Acidic soils and Al-stress resistance for both symbionts and plants were likely to influence the
471 survival and growth of legumes. Yang et al. 2012 described that an Al-exclusion mechanism
472 in soybean cultivars was conferred by enhancing a specific Al-induced exudation of citrate.
473 Accordingly, citric and malic acids had a positive correlation with bacterial communities,
474 suggesting that citric and malic acid may be positive selection forces with some microbes in
475 the rhizosphere soil (Yang et al 2012). Therefore, diversity of soils and climates ensures a va-

riety of native bacteria adapting to these diverse conditions. Moreover, the nitrogen-fixing capacity of bacteria might significantly differ between rhizobia and edapho-climate. In addition, research about Al-tolerance under different pH conditions is essential to maximize symbiotic efficiency, allowing a differentiation between tolerant and sensitive varieties that could establish good interaction with beneficial microorganisms. It is necessary to continue studies to evaluate symbiotic effectiveness and tolerance to adverse environmental factors. In addition, pathways of Al-acid resistance of the soybean 'INIA' requires further analysis. Consequently, these suggestions can help to solve food problem in areas with abiotic stress conditions.

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Figures captions

Fig. 1. The effect of aluminum on cell growth and viability. Cell growth rate of α-rhizobia, (A) *Bradyrhizobium diazoefficiens* USDA 110 and *Rhizobium pusense* VAF1243 (B). Cell growth rate of β-rhizobia, (C) *Burkholderia fungorum* VTr35 and *Burkholderia symbiont* VG10B. The control condition was 0 mM of Al (..... Log. tendency line).

Fig. 2. Nodulation activity under different Al-stress conditions. A) Number the nodules obtained at 15 days using *G. max* 'INIA'. B) Nodulation activity at 15 days using *G. max* 'Enrei'. C) The number of nodules obtained at 30 days using *G. max* 'INIA'. D) Nodulation activity at 30 days using *G. max* 'Enrei'.

Fig. 3. Morphological characterization of *in vitro* plant test using *Bradyrhizobium diazoefficiens* USDA 110 and *Burkholderia fungorum* VTr35 under different acid-Al stress conditions. A) Leaves taken next to the primordium. B) Roots appearances after 30 days. C) Internal appearance of nodules under stress conditions according to the type of rhizobia, respectively. *This figure is referred to plant test with soybean cultivar INIA under 4.5 pH.

Fig. 4. The relation between biomass and type of rhizobia after 15 days *in vitro* cultivation. A) Biomass using *G. max* 'INIA' as host trap. B) Biomass using *G. max* 'Enrei'.

Fig. 5. Acetylene reduction activity (ARA) after 15 days cultivation under Al-stress conditions. A) Biomass *G. max* 'INIA' as host trap. B) Biomass using *G. max* 'Enrei'.

Table 1. Tolerance to different concentrations of Aluminum at two pH conditions

Aluminium ($AlCl^3$ mM)	0		0.1		0.5		1.0		2.0	
pH	**4.5**	**6.8**	**4.5**	**6.8**	**4.5**	**6.8**	**4.5**	**6.8**	**4.5**	**6.8**
***Bradyrhizobium diazoefficiens* USDA 110**	++	+++	+++	+++	++	+++	+	++	-	+
***Rhizobium pusense* VAF1243**	++	+++	++	+++	++	+++	+	+++	-	+++
***Burkholderia fungorum* VTr35**	+++	+++	+++	+++	+++	+++	++	++	+	++
***Burkholderia symbiont* VG10B**	++	+++	++	+++	+	+++	+	+++	-	+++

Measures as, no growth: (-); weak growth: (+) 10-20%; good growth: (++) 30-60%; very good or same to control: (+++) 100% growth (compared with the control)

Table 2. Released organic acids and IAA production by rhizobia under Al-stress conditions

Strain	pH	Al^{3+} (mM)	Citric acid (mg/ml)*	Tartaric acid (mg/ml)	Malic acid (mg/ml)***	Succinic acid (mg/ml)**	Fumaric acid (mg/ml)	IAA (µg/ml)
B. diazoefficiens USDA 110	6.8	0	0.060	0.001	0.042	0.287	0.000	1.21
		0.1	0.650	0.000	0.037	0.240	0.018	0.24
		0.5	0.677	0.000	0.046	0.243	0.014	0.00
		1	0.673	0.000	0.024	0.218	0.003	0.00
		2	0.682	0.000	0.037	0.253	0.003	0.00
	4.5	0	0.309	0.001	0.014	0.083	0.017	0.00
		0.1	0.297	0.001	0.012	0.078	0.016	0.00
		0.5	0.330	0.001	0.023	0.122	0.019	0.00
		1	0.162	0.001	0.010	0.264	0.021	0.00
		2	0.197	0.001	0.011	0.066	0.003	0.00
*R. pusense VAF*1243	6.8	0	0.221	0.000	0.014	0.000	0.002	1.22
		0.1	0.208	0.002	0.013	0.583	0.005	0.17
		0.5	0.172	0.000	0.009	0.454	0.004	0.00
		1	0.162	0.000	0.007	0.421	0.003	0.00
		2	0.152	0.000	0.005	0.321	0.002	0.00
	4.5	0	0.195	0.000	0.025	0.000	0.002	0.00
		0.1	0.247	0.008	0.017	0.236	0.006	0.00
		0.5	0.215	0.005	0.016	0.096	0.009	0.00
		1	0.209	0.003	0.013	0.081	0.004	0.00
		2	0.128	0.000	0.013	0.000	0.000	0.00
B. fungorum VTr35	6.8	0	0.386	0.042	0.049	0.000	0.000	1.79
		0.1	0.878	0.000	0.043	0.000	0.026	1.36*
		0.5	0.843	0.004	0.025	0.748	0.030	0.64*
		1	0.679	0.002	0.022	0.192	0.042	0.21*
		2	0.633	0.000	0.028	0.092	0.027	0.00
	4.5	0	0.805	0.004	0.051	0.000	0.040	0.00
		0.1	0.349	0.001	0.040	0.000	0.026	0.00
		0.5	0.368	0.001	0.041	0.000	0.026	0.00
		1	0.355	0.001	0.042	0.000	0.028	0.00
		2	0.443	0.001	0.034	0.000	0.030	0.00
B. symbiont VG10B	6.8	0	0.061	0.000	0.011	0.000	0.000	1.07
		0.1	0.126	0.000	0.013	0.588	0.011	0.29
		0.5	0.122	0.012	0.008	0.445	0.002	0.14*
		1	0.104	0.010	0.005	0.441	0.002	0.00
		2	0.092	0.000	0.005	0.000	0.000	0.00
	4.5	0	0.175	0.000	0.015	0.000	0.001	0.00
		0.1	0.248	0.011	0.011	0.000	0.011	0.00
		0.5	0.216	0.005	0.005	0.000	0.010	0.00
		1	0.212	0.005	0.002	0.000	0.030	0.00
		2	0.219	0.000	0.002	0.000	0.000	0.00

(*) significant result $p < 0.05$

Table 3. Nitrogen fixation activity and biomass using *G. max* 'Enrei' under *in vitro* Al-stress conditions

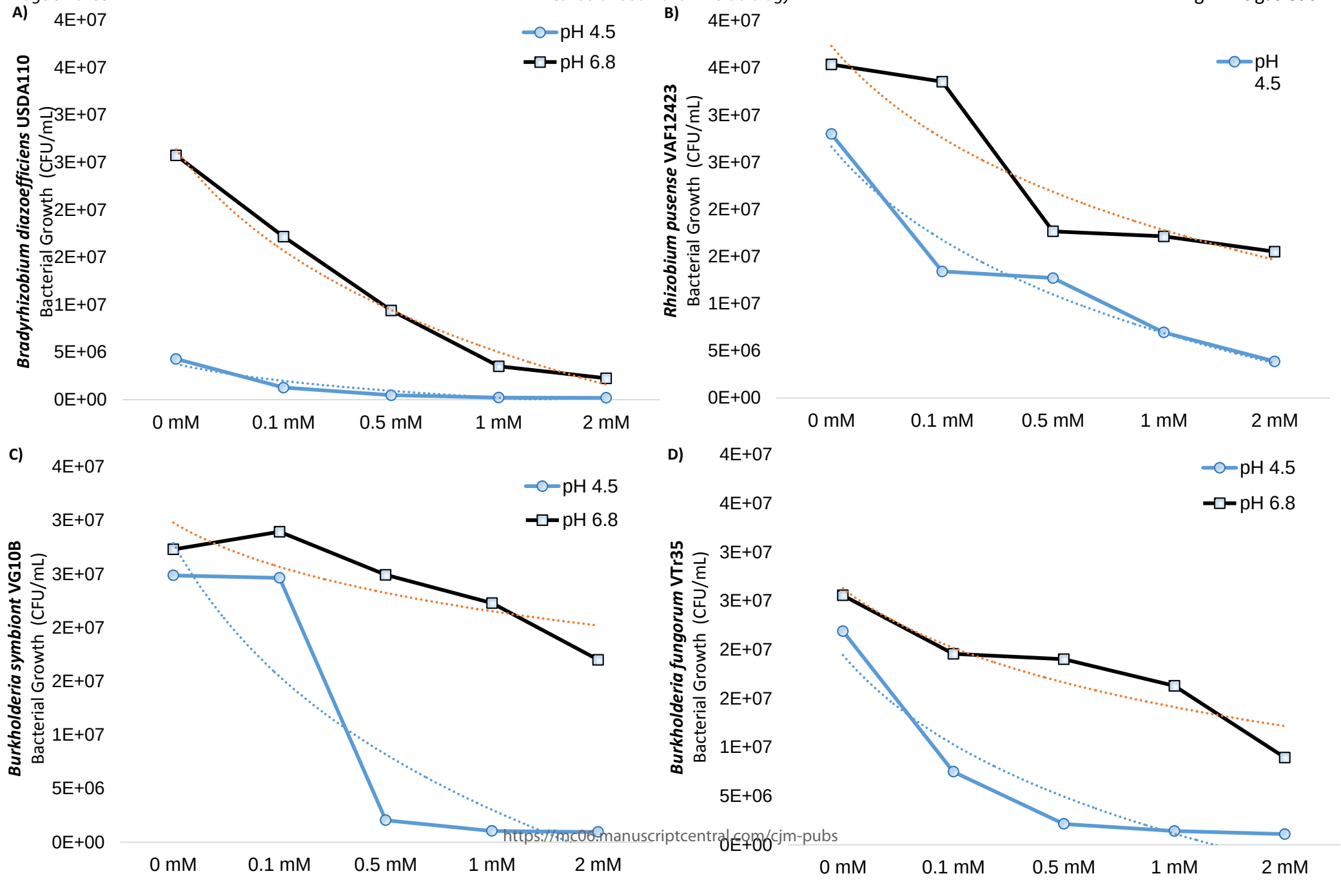
Conditions			Length (cm)		Dry Weight (g)		ARA (µM)
Type of Rhizobia	pH	Al (mM)	Root	Shoot	Root	Shoot	DW g plant^{-1}
Control (No Inoculation)	6.8	0	14.00 ± 0.2	20.00 ± 0.6	0.14 ± 0.03	0.30 ± 0.09	0.00
		0.1	13.00 ± 0.3	18.00 ± 0.3	0.15 ± 0.02	0.30 ± 0.07	0.00
		0.5	13.00 ± 0.5	16.00 ± 0.4	0.13 ± 0.04	0.29 ± 0.04	0.00
		1	10.00 ± 0.6	15.33 ± 0.9	0.08 ± 0.01	0.10 ± 0.02	0.00
		2	9.30 ± 0.7	14.00 ± 0.2	0.084 ± 0.01	0.10 ± 0.01	0.00
	4.5	0	13.33 ± 0.2	18.00 ± 0.2	0.14 ± 0.02	0.30 ± 0.05	0.00
		0.1	13.00 ± 0.3	16.00 ± 0.3	0.13 ± 0.02	0.29 ± 0.03	0.00
		0.5	10.33 ± 0.4	15.33 ± 0.5	0.08 ± 0.01	0.10 ± 0.02	0.00
		1	9.33 ± 0.7	14.00 ± 0.1	0.075 ± 0.01	0.09 ± 0.01	0.00
		2	8.33 ± 0.9	12.00 ± 0.1	0.07 ± 0.01	0.09 ± 0.02	0.00
B. diazoefficiens USDA 110	6.8	0	15.67 ± 1.6	35.00 ± 5.0	0.11 ± 0.03	0.44 ± 0.04	2.45 ± 0.15*
		0.1	15.33 ± 1.4	29.00 ± 6.6	0.14 ± 0.08	0.41 ± 0.09	1.30 ± 1.0*
		0.5	15.33 ± 1.5	22.33 ± 3.2	0.22 ± 0.16	0.47 ± 0.02	0.04 ± 0.03
		1	14.00 ± 1.0	21.00 ± 3.6	0.11 ± 0.02	0.33 ± 0.02*	0.01 ± 0.01**
		2	11.67 ± 0.6	20.33 ± 2.5	0.08 ± 0.01	0.28 ± 0.04*	0.00
	4.5	0	14.33 ± 0.6	29.33 ± 1.2	0.25 ± 0.06	0.41 ± 0.03	0.19 ± 0.05
		0.1	14.00 ± 1.0	28.33 ± 2.9	0.18 ± 0.08	0.38 ± 0.05	0.06 ± 0.04
		0.5	14.50 ± 0.5	23.33 ± 1.5	0.16 ± 0.05	0.31 ± 0.01	0.00
		1	13.33 ± 1.5	22.67 ± 1.2	0.11 ± 0.01	0.22 ± 0.07	0.00
		2	11.00 ± 1.0	19.00 ± 1.0	0.09 ± 0.01	0.14 ± 0.01	0.00
R. pusense VAF1243	6.8	0	15.67 ± 0.6	38.00 ± 2.0	0.17 ± 0.04	0.41 ± 0.02	0.38 ± 0.24
		0.1	14.33 ± 0.4	30.33 ± 0.6	0.17 ± 0.04	0.36 ± 0.05	0.11 ± 0.06
		0.5	14.17 ± 0.3	25.00 ± 1.0	0.13 ± 0.03	0.24 ± 0.04	0.02 ± 0.02
		1	11.67 ± 0.6	21.33 ± 0.6	0.11 ± 0.02	0.19 ± 0.01	0.00
		2	9.33 ± 1.2	19.00 ± 1.0	0.08 ± 0.01	0.12 ± 0.01	0.00
	4.5	0	19.00 ± 2.6	24.33 ± 3.2	0.14 ± 0.03	0.36 ± 0.05	0.45 ± 0.16
		0.1	14.00 ± 1.0	22.33 ± 0.6	0.13 ± 0.03	0.33 ± 0.08	0.05 ± 0.04
		0.5	13.33 ± 0.6	24.33 ± 0.7	0.10 ± 0.02	0.23 ± 0.02	0.00
		1	11.33 ± 0.6	18.33 ± 1.5	0.03 ± 0.01	0.16 ± 0.01	0.00
		2	10.33 ± 0.6	15.67 ± 0.4	0.02 ± 0.01	0.10 ± 0.02	0.00
B. fungorum VTr35	6.8	0	16.67 ± 1.5	34.33 ± 9.0	0.12 ± 0.05	0.61 ± 0.19	2.01 ± 0.23*
		0.1	14.33 ± 0.6	32.17 ± 1.8	0.15 ± 0.10	0.46 ± 0.09	1.12 ± 0.9*
		0.5	14.00 ± 1.0	27.67 ± 2.5	0.12 ± 0.06	0.41 ± 0.12	0.11 ± 0.05
		1	12.67 ± 0.6	24.67 ± 0.6	0.10 ± 0.03	0.32 ± 0.03*	0.12 ± 0.06**
		2	11.67 ± 0.6	19.67 ± 0.6	0.06 ± 0.01	0.28 ± 0.04*	0.03 ± 0.01**
	4.5	0	14.00 ± 1.0	29.33 ± 0.5	0.22 ± 0.09	0.46 ± 0.05	1.46 ± 0.1
		0.1	13.67 ± 0.6	28.33 ± 2.9	0.11 ± 0.04	0.41 ± 0.01	0.12 ± 0.01
		0.5	13.67 ± 0.6	26.00 ± 1.7	0.09 ± 0.02	0.34 ± 0.04	0.10 ± 0.01
		1	12.67 ± 0.6	23.67 ± 1.5	0.04 ± 0.01	0.28 ± 0.03	0.07 ± 0.02
		2	11.67 ± 0.6	19.67 ± 0.6	0.03 ± 0.01	0.22 ± 0.02*	0.03 ± 0.01*
B. symbiont VG10B	6.8	0	15.67 ± 0.7	35.00 ± 1.7	0.20 ± 0.02	0.42 ± 0.01	0.35 ± 0.04
		0.1	14.00 ± 1.1	27.67 ± 2.1	0.16 ± 0.04	0.32 ± 0.03	0.23 ± 0.09
		0.5	12.67 ± 0.7	21.33 ± 0.6	0.11 ± 0.01	0.26 ± 0.05	0.00
		1	11.67 ± 0.5	21.33 ± 0.7	0.14 ± 0.02	0.20 ± 0.02	0.00
		2	9.33 ± 1.2	19.00 ± 1.0	0.05 ± 0.01	0.12 ± 0.01	0.00
	4.5	0	15.33 ± 0.4	28.00 ± 1.1	0.14 ± 0.03	0.38 ± 0.01	0.19 ± 0.04
		0.1	14.00 ± 1.0	24.50 ± 0.5	0.10 ± 0.01	0.30 ± 0.09	0.18 ± 0.06
		0.5	14.00 ± 1.0	21.00 ± 1.1	0.02 ± 0.02	0.20 ± 0.01	0.00
		1	10.33 ± 0.6	17.00 ± 0.9	0.03 ± 0.02	0.14 ± 0.03	0.00
		2	8.33 ± 1.3	15.33 ± 0.5	0.02 ± 0.01	0.10 ± 0.01	0.00

*significant result under stress conditions

Table 4. Nitrogen fixation activity and biomass using *G. max* 'INIA' under *in vitro* Al-stress conditions

Conditions			Length (cm)		Dry Weight (g)		ARA (μM)
Type of Rhizobia	pH	Al (mM)	Root	Shoot	Root	Shoot	DW g plant^{-1}
Control (No Inoculation)	6.8	0	14.00 ± 0.8	19.00 ± 1.0	0.15 ± 0.02	0.29 ± 0.05	0.00
		0.1	15.00 ± 1.0	18.00 ± 0.5	0.15 ± 0.01	0.31 ± 0.06	0.00
		0.5	12.00 ± 0.2	15.00 ± 0.1	0.12 ± 0.02	0.28 ± 0.03	0.00
		1	10.00 ± 1.0	14.00 ± 0.5	0.09 ± 0.02	0.10 ± 0.03	0.00
		2	9.00 ± 0.5	13.00 ± 0.1	0.09 ± 0.01	0.089 ± 0.01	0.00
	4.5	0	14.30 ± 0.3	18.00 ± 0.3	0.15 ± 0.03	0.29 ± 0.02	0.00
		0.1	14.00 ± 0.1	16.00 ± 0.2	0.13 ± 0.03	0.28 ± 0.02	0.00
		0.5	11.00 ± 0.5	15.00 ± 0.4	0.09 ± 0.02	0.12 ± 0.03	0.00
		1	9.53 ± 0.2	13.00 ± 0.2	0.085 ± 0.01	0.090 ± 0.01	0.00
		2	9.00 ± 0.9	12.00 ± 0.1	0.083 ± 0.01	0.084 ± 0.01	0.00
B. diazoefficiens USDA 110	6.8	0	17.33 ± 0.6	28.67 ± 1.5	0.22 ± 0.07	0.58 ± 0.03	2.33 ± 0.24*
		0.1	18.00 ± 1.0	28.80 ± 1.0	0.22 ± 0.02	0.45 ± 0.05	1.92 ± 0.66*
		0.5	17.00 ± 0.1	21.33 ± 0.6	0.16 ± 0.01	0.45 ± 0.02	1.90 ± 0.20*
		1	15.33 ± 0.6	18.33 ± 0.6	0.13 ± 0.01	0.31 ± 0.02*	1.28 ± 0.25*
		2	15.00 ± 0.5	16.33 ± 0.8	0.09 ± 0.02	0.23 ± 0.03*	0.75 ± 0.65*
	4.5	0	16.00 ± 1.0	26.67 ± 2.9	0.21 ± 0.02	0.38 ± 0.03	0.49 ± 0.01**
		0.1	25.00 ± 1.0	32.00 ± 2.0	0.12 ± 0.06	0.31 ± 0.02	0.62 ± 0.28**
		0.5	16.00 ± 0.9	22.33 ± 0.6	0.10 ± 0.02	0.38 ± 0.01	0.45 ± 0.14**
		1	13.33 ± 0.6	21.67 ± 0.6	0.07 ± 0.02	0.25 ± 0.04	0.05 ± 0.02**
		2	11.00 ± 0.8	17.00 ± 0.8	0.03 ± 0.01	0.13 ± 0.01	0.02 ± 0.01**
R. pusense VAF1243	6.8	0	16.00 ± 0.9	36.33 ± 1.5	0.16 ± 0.03	0.71 ± 0.09	0.90 ± 0.26
		0.1	14.63 ± 0.6	31.00 ± 1.0	0.12 ± 0.04	0.60 ± 0.01	0.29 ± 0.06
		0.5	14.07 ± 0.2	25.80 ± 0.9	0.07 ± 0.03	0.29 ± 0.04	0.13 ± 0.10
		1	11.00 ± 0.4	21.00 ± 0.5	0.06 ± 0.02	0.19 ± 0.01	0.00
		2	9.23 ± 1.5	19.45 ± 0.8	0.02 ± 0.02	0.16 ± 0.01	0.00
	4.5	0	20.00 ± 5.2	24.00 ± 2.6	0.10 ± 0.02	0.48 ± 0.03	0.28 ± 0.10
		0.1	14.50 ± 0.9	23.00 ± 1.7	0.09 ± 0.02	0.30 ± 0.02	0.24 ± 0.04
		0.5	13.00 ± 0.5	23.90 ± 0.6	0.05 ± 0.01	0.22 ± 0.01	0.00
		1	11.00 ± 0.4	18.00 ± 0.5	0.05 ± 0.02	0.16 ± 0.02	0.00
		2	10.13 ± 0.1	14.90 ± 0.6	0.02 ± 0.008	0.11 ± 0.01	0.00
B. fungorum VTr35	6.8	0	24.33 ± 0.6	29.33 ± 1.0	0.20 ± 0.04	0.93 ± 0.09	1.98 ± 0.30*
		0.1	25.33 ± 1.2	23.67 ± 0.6	0.23 ± 0.03	0.66 ± 0.05	2.04 ± 0.01*
		0.5	24.30 ± 0.5	22.67 ± 0.5	0.23 ± 0.02	0.60 ± 0.06	1.79 ± 0.02*
		1	22.67 ± 0.6	21.33 ± 0.3	0.17 ± 0.03	0.51 ± 0.02*	1.56 ± 0.10*
		2	20.67 ± 0.7	20.33 ± 0.6	0.11 ± 0.01	0.43 ± 0.03*	0.76 ± 0.01*
	4.5	0	23.33 ± 0.6	32.33 ± 2.6	0.34 ± 0.05	0.94 ± 0.05	1.37 ± 0.01*
		0.1	18.50 ± 0.5	29.00 ± 2.0	0.21 ± 0.02	0.78 ± 0.07	1.40 ± 0.09*
		0.5	17.67 ± 0.8	26.00 ± 1.7	0.18 ± 0.01	0.51 ± 0.02	0.90 ± 0.01*
		1	16.07 ± 1.0	24.33 ± 0.6	0.17 ± 0.02	0.44 ± 0.03*	0.82 ± 0.05*
		2	15.67 ± 0.7	23.17 ± 0.8	0.12 ± 0.008	0.35 ± 0.05*	0.24 ± 0.01*
B. symbiont VG10B	6.8	0	14.67 ± 0.5	33.00 ± 3.7	0.18 ± 0.03	0.48 ± 0.03	0.61 ± 0.06
		0.1	14.00 ± 0.9	27.00 ± 1.2	0.09 ± 0.02	0.30 ± 0.02	0.37 ± 0.19
		0.5	12.00 ± 0.6	21.00 ± 0.6	0.06 ± 0.01	0.24 ± 0.01	0.15 ± 0.09
		1	11.07 ± 0.6	20.90 ± 0.5	0.06 ± 0.02	0.20 ± 0.05	0.00
		2	9.67 ± 1.6	18.90 ± 1.0	0.05 ± 0.01	0.11 ± 0.01	0.00
	4.5	0	15.00 ± 0.2	27.00 ± 1.5	0.12 ± 0.03	0.40 ± 0.07	0.21 ± 0.03
		0.1	14.00 ± 0.4	25.17 ± 0.8	0.10 ± 0.01	0.33 ± 0.08	0.19 ± 0.01
		0.5	13.85 ± 1.0	21.00 ± 0.9	0.03 ± 0.02	0.23 ± 0.02	0.00
		1	10.00 ± 0.2	16.50 ± 0.8	0.02 ± 0.009	0.16 ± 0.01	0.00
		2	8.00 ± 1.3	15.00 ± 0.6	0.02 ± 0.008	0.10 ± 0.02	0.00

*significant result under stress conditions



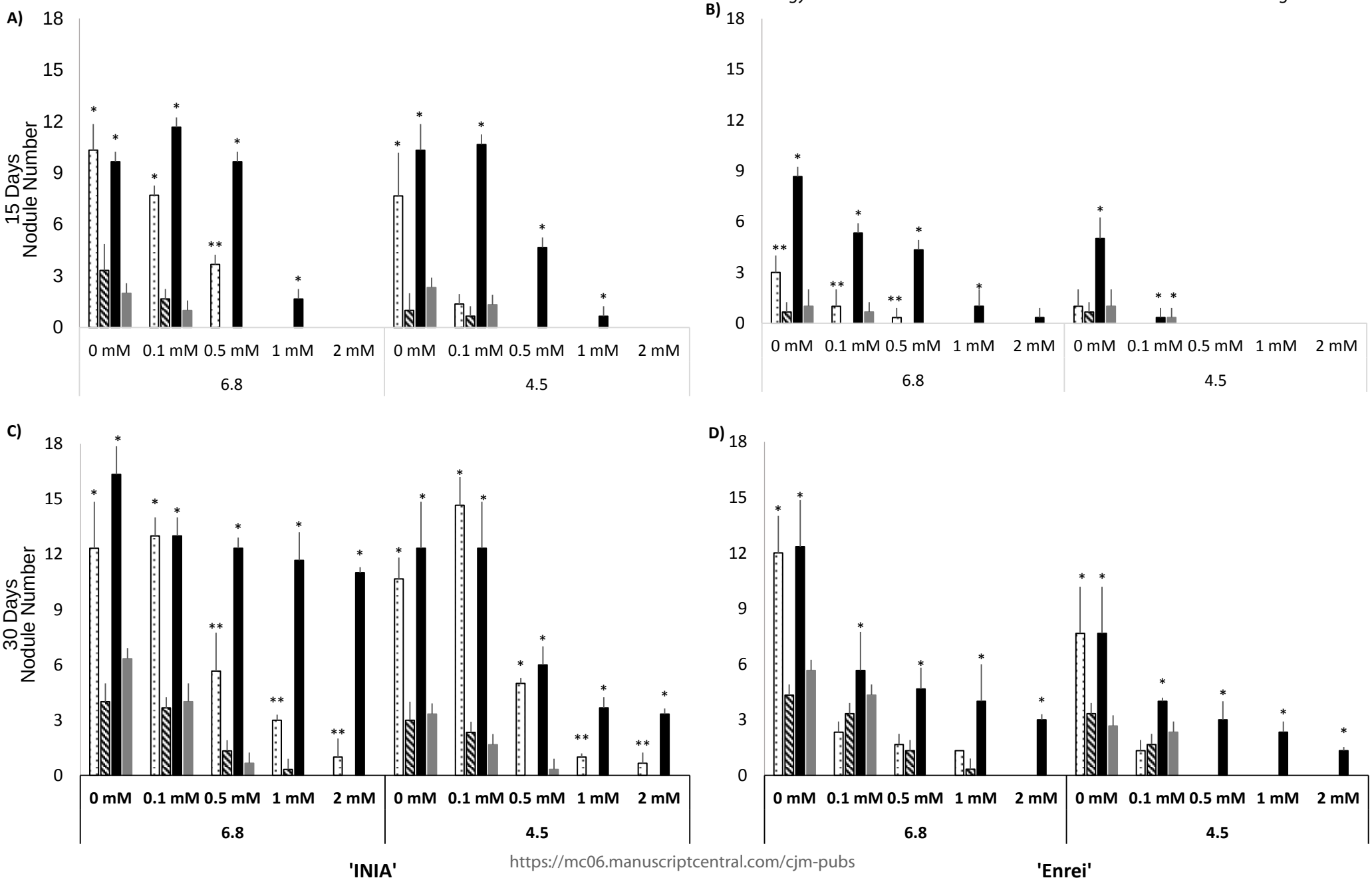
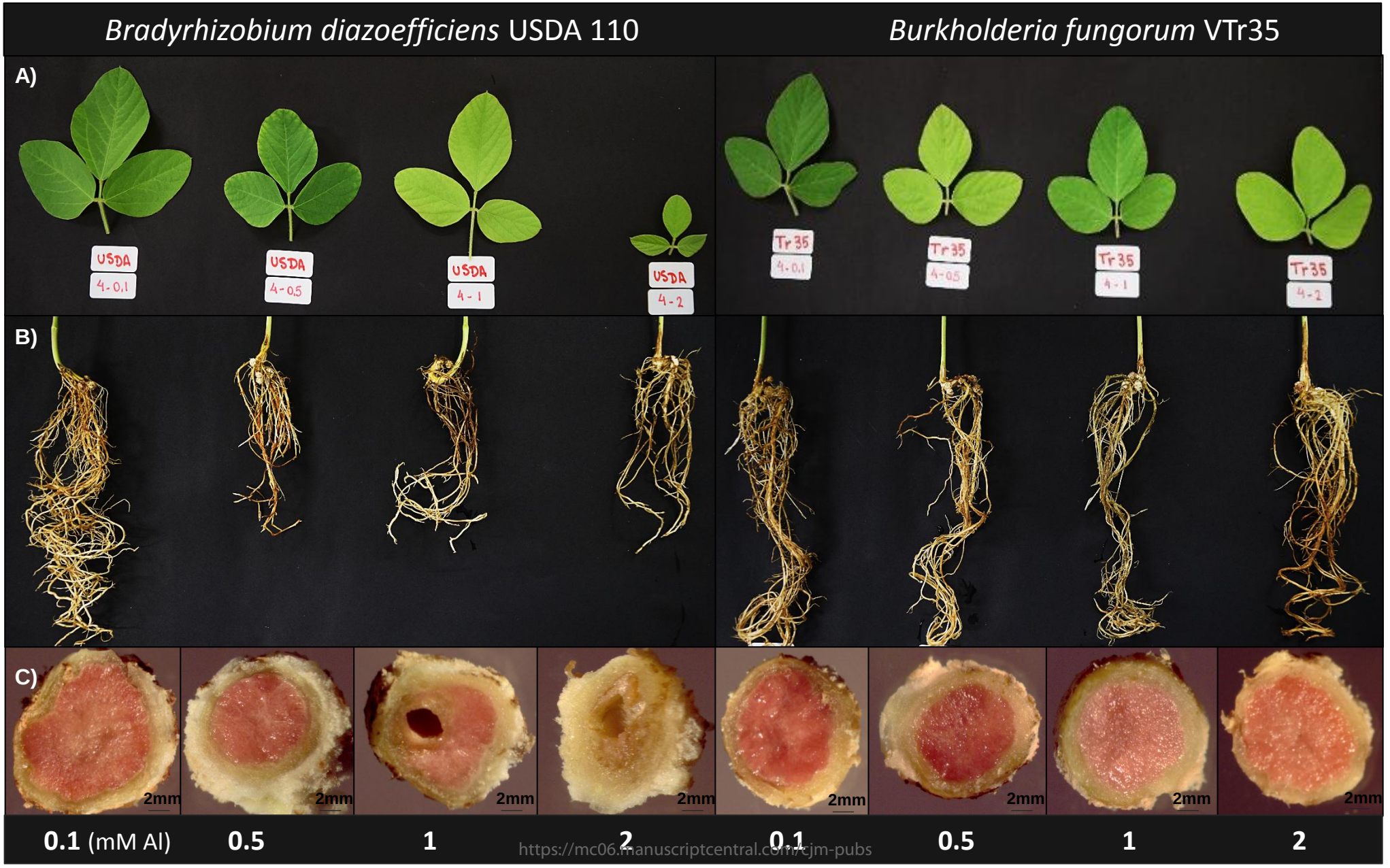
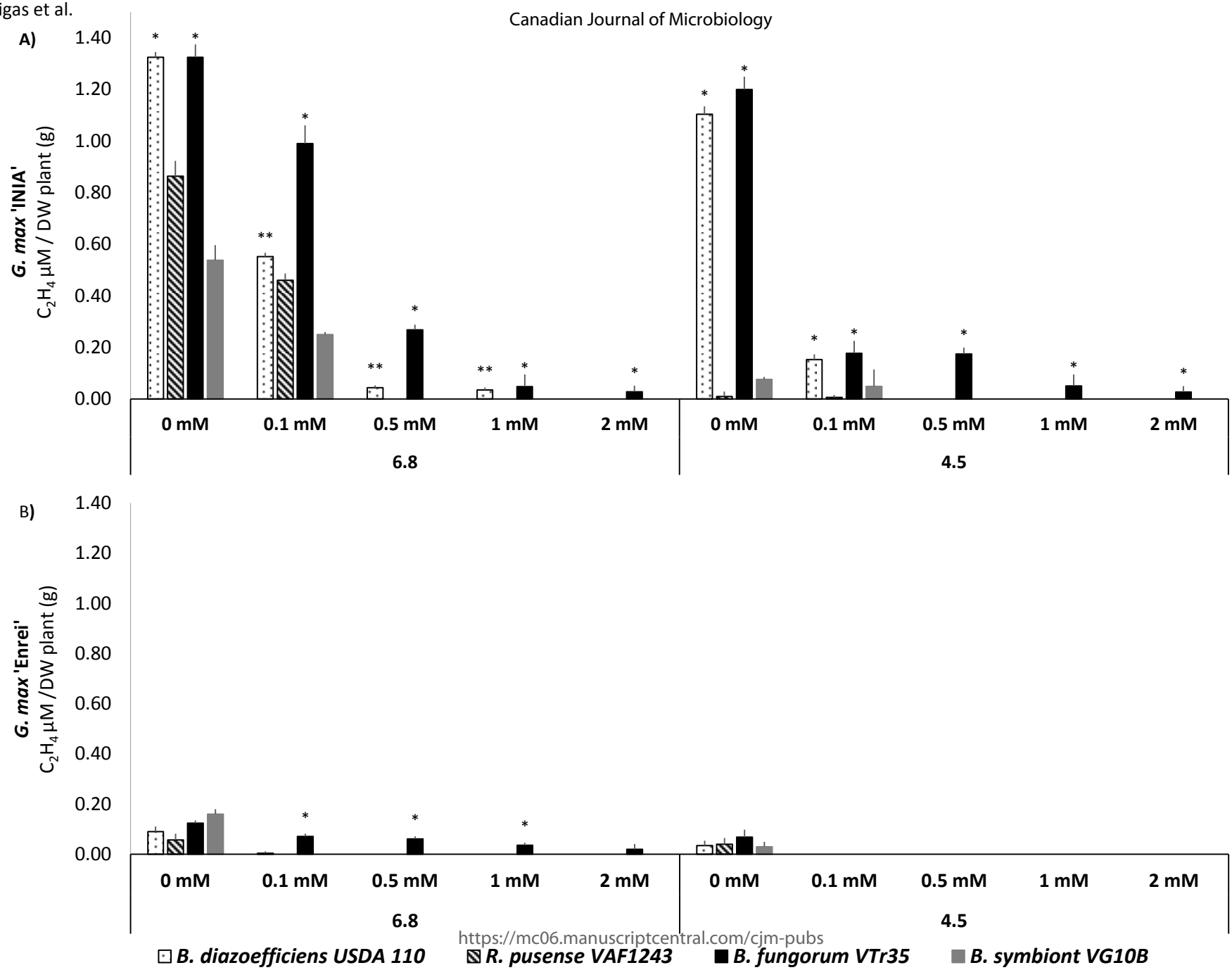
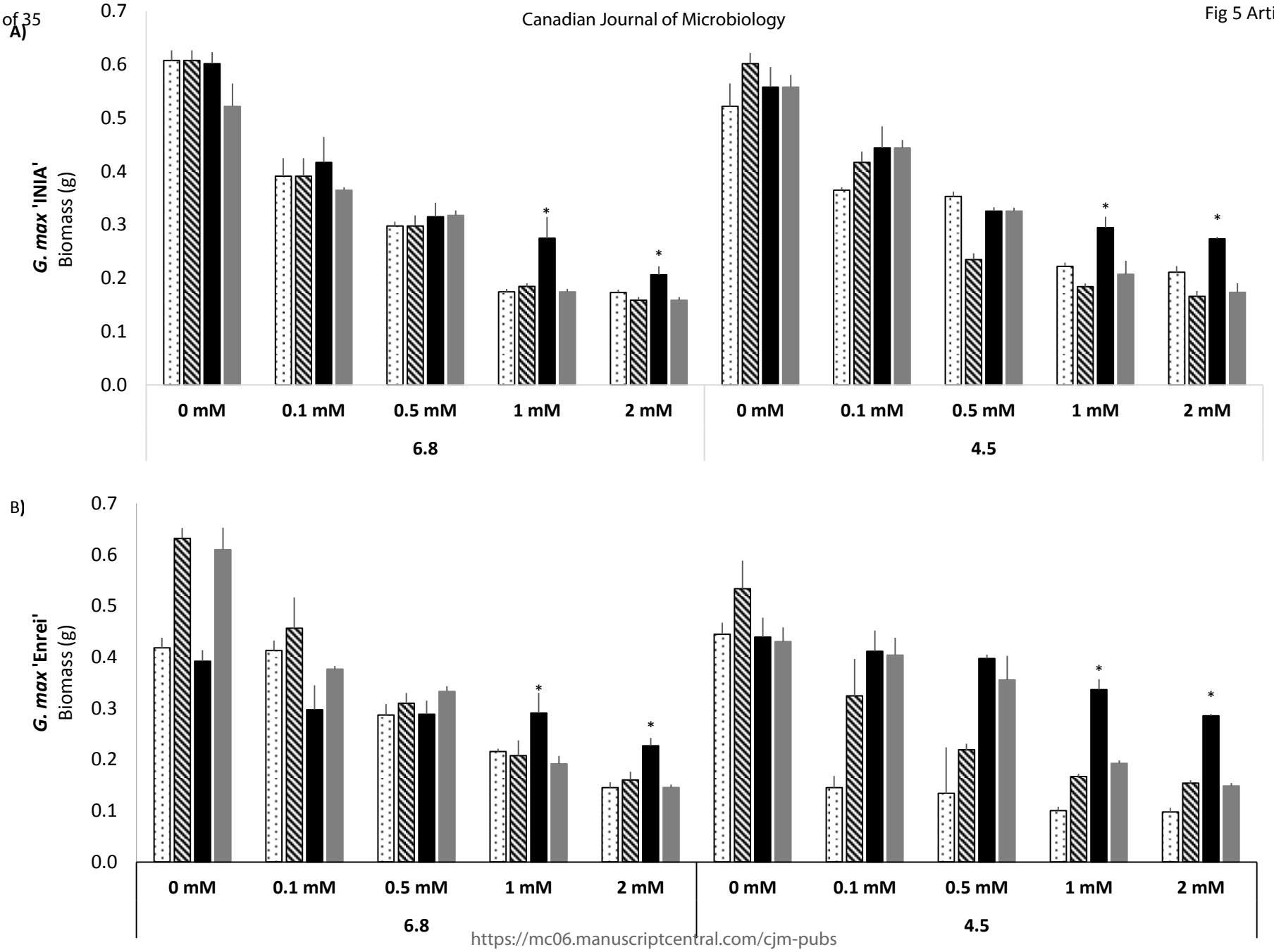

A)
B)
C)
D)
15 Days Nodule Number
30 Days Nodule Number
0 mM
0.1 mM
0.5 mM
1 mM
2 mM
6.8
4.5
'INIA'
'Enrei'
B. diazoefficiens USDA 110
R. pusense VAF1243
B. fungorum VTr35
B. symbiont VG10B

Fig 3 Artigas et al.




A)
G. max 'INIA'
C2H4 µM / DW plant (g)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0 mM
0.1 mM
0.5 mM
1 mM
2 mM
6.8
4.5
B)
G. max 'Enrei'
C2H4 µM /DW plant (g)
B. diazoefficiens USDA 110
R. pusense VAF1243
B. fungorum VTr35
B. symbiont VG10B


A)
G. max 'INIA' Biomass (g)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 mM
0.1 mM
0.5 mM
1 mM
2 mM
6.8
4.5
B)
G. max 'Enrei' Biomass (g)
https://mc06.manuscriptcentral.com/cjm-pubs
B. diazoefficiens USDA 110
R. pusense VAF1243
B. fungorum VTr35
B. symbiont VG10B