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PARAQUAT TOXICITY ON THE GROWTH OF *RHIZOBIUM* Sp. IN A SYNTHETIC MEDIUM

TOKSISITAS PARAKUAT TERHADAP PERTUMBUHAN RHIZOBIUM *Sp. DALAM MEDIUM SINTETIK.*

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INTISARI

Parakuat merupakan bahan aktif beberapa jenis herbisida yang diaplikasikan secara luas dan rutin di lahan gambut maupun di lahan tadah hujan. Studi ini dilakukan untuk mengetahui toksisitas parakuat terhadap pertumbuhan Rhizobium *sp. dalam medium* Yeast Extract Mannitol. *Beberapa strain* Rhizobium *diuji secara semi-kualitatif menggunakan teknik difusi cakram kertas* (paper disc diffusion technique). *Selanjutnya toksisitas parakuat terhadap pertumbuhan* Rhizobium *dalam medium sintetik diuji secara kuantitatif menggunakan metoda* plating.

Hasil uji kualitatif menunjukkan bahwa toleransi Rhizobium *sp. terhadap parakuat bervariasi tergantung pada strainnya. Beberapa strain tahan terhadap parakuat hingga 100 ppm, tetapi strain lainnya sudah terhambat pada konsentrasi 20 ppm. Pengamatan terhadap pertumbuhan sel* Rhizobium *sp. menunjukkan bahwa pengaruh paraquat tergantung pada konsentrasinya. Beberapa strain tahan terhadap parakuat konsentrasi 20 dan 40 ppm. Pada konsentrasi ini, jumlah sel meningkat dari 10^6 menjadi 10^7 atau 10^8* CFU/mL. *Angka ini lebih rendah dibandingkan dalam medium tanpa parakuat, di mana terdeteksi adanya pertumbuhan yang mencapai tingkat 10^8 atau 10^9* CFU/mL. *Namun demikian, pada konsentrasi parakuat yang lebih tinggi (100 ppm) strain G-69 dan G-182 turun dari 10^6 menjadi 10^4* CFU/m. *Sedangkan* R. japonicum *strain 143 dan* Rhizobium *sp. strain KS mengalami kematian, dan jumlah sel menurun secara signifikan hingga 10^1* CFU/mL *hanya dalam waktu inkubasi 8 hari.*

Kenyataan bahwa paraquat mengakibatkan penurunan populasi Rhizobium, *menimbulkan kekhawatiran. Karena jumlah sel yang rendah akan mengurangi kemungkinan terbentuknya bintil akar dan terjadinya fiksasi nitrogen pada tanaman leguminosa. Pada gilirannya hal ini dapat mempengaruhi pertumbuhan dan produksi tanaman. Mengingat makin meluasnya pemakaian herbisida dengan bahan aktif parakuat di lahan gambut dan di lahan pertanian tadah hujan di Indonesia; serta peran* Rhizobium *dalam fiksasi nitrogen, hasil penelitian ini memiliki arti penting terutama untuk mengurangi dampak negatif aplikasi parakuat terhadap mikrobia tanah.*

Kata-kata kunci : toksisitas parakuat, Rhizobium *sp., medium sintetik.*

ABSTRACT

Toxicity of paraquat on the growth of several strains of *Rhizobium* sp. in Yeast Extract Mannitol medium was studied. Various concentrations of paraquat ion, ranged from 0 (control) to 100 ppm were applied. Qualitative examination was done using paper disc diffusion technique, and the quantitative examination was conducted based on the change in cell density in medium measured by plate count method.

Qualitative data showed that effect of paraquat was species specific. Some strains of *Rhizobium* sp., namely *Rhizobium* sp. strain T-37 and QF, were tolerant to paraquat until 100 ppm, but other strains were sensitive to paraquat, especially at high concentration. Quantitative examination to the sensitive strains shows that higher concentration of paraquat caused higher toxicity to the growth of *Rhizobium. Rhizobium* sp. strains G-69 and G-182, paraquat addition at100 ppm slightly decreased cell density from 10^6 to 10^4 CFU/mL.

Rhizobium japonicum strains 143 and KS were tolerant to 20 and 40 ppm of paraquat; their cell density increased from 10^6 to a level of 10^7 or 10^8 CFU/mL depend on the strain. This level was not significantly lower than those in medium without paraquat, in which maximal population density reached to 10^8 or 10^9 CFU/mL. Addition of higher paraquat concentration damaged the cell of these strains, and caused population density decreased significantly to a level of 10^1 CFU/mL.

The data which show that paraquat was toxic to *Rhizobium* sp. were important, because growth inhibition of these bacteria may influence the formation of root nodule on leguminous plants, and in turn will decrease the yield. Due to widely applied paraquat in agricultural and plantation systems, and the role of *Rhizobium* in nitrogen fixation, these results are important for minimizing the impacts of paraquat application.

Key words: paraquat toxicity, *Rhizobium* sp., synthetic medium.

INTRODUCTION

Weeds are the most severe and widespread biological constraint to agriculture, especially in rice production, in which the yield reduction were estimated to be in the range of 45 – 95%, depending on agricultural system and environmental factors (Naylor, 1996). Many kinds of herbicides were applied periodically for controlling the weed growth. However, it was reported that some herbicides decreased soil and water quality, soil productivity, inhibited the growth of crops and soil microorganisms (Anderson, 1978). Rao (1994) insisted that herbicides decreased the population of *Azotobacter, Rhizobium, Nitrosomonas, Nitrobacter,* cellulolytic and also phosphate-solubilizing microorganisms. All of these microorganisms are important in nutrient cycle and soil health. Disturbances of microbial population may cause the decrease of environmental quality and soil fertility.

Paraquat is an active agent of some herbicides widely used in peat land, rain-fed and zero-tillage agricultural systems. Biederbeck *et al.*, (1993) reported that repeated application of paraquat in a dark brown soil did not influence ammonifier and nitrifier microbial population. These data were supported by Katayama and Kuwatsuka (1992), which found microorganisms tolerant to paraquat until 1000 ppm. However, other studies showed that paraquat influenced the growth of soil microorganisms (Anderson, 1978; Setyaningsih *et al.*, 2001). Addition of paraquat at 20 ppm in peat soil changed the population dynamics of soil bacteria and fungi (Margino *et al.*, 2000), or nitrifying bacteria and phosphate-subsidizer (Setyaningsih *et al., 2001*). Paraquat also influenced Nitrogen fixing bacteria of *Azotobacter* sp. (Anderson, 1978) and *Rhizobium* sp. (Martani *et al.*, 2001b).

Rhizobium sp. is important not only in symbiotic nitrogen fixation with legumes, but also in plant resistance to plant pathogens and herbivores (Hammerschmidt & Smith-Becker, 1999; Karban & Kue, 1999). Therefore, disturbance of paraquat on the growth of *Rhizobium.* sp. or *Bradyrhizobium* sp. might also be responsible to the growth inhibition, reduction the number of root nodule and yiled of soybean in peat soil (Martani *et al.* 2001a).

This research was conducted to know the influence of paraquat on the growth of *Rhizobium* sp. in synthetic medium. The experiments were done in laboratory level. Results of this study will be important because there was a minimum density level for *Rhizobium* to form root nodule and fix free nitrogen (Vincent, 1970).

MATERIALS AND METHODS

Paraquat herbicide. Gramoxone® (Zeneca corp.) was used in this research. The paraquat concentration in gramoxone was 200 mg of paraquat dichloride per Liter.

The cultures of* Rhizobium *sp. Several strains of *Rhizobium* were used in this study. They were obtained from some Microbial Culture Collection Institutes, or isolated from peat soil using Yeast-Extract Mannitol Agar (YMA) medium added with Congo-red at 1% w/v (Martani *et al.*, 2001b). The composition of this medium is (g/L) mannitol 10.0; K_2HPO_4 0.5; $MgSO_4.7H_2O$ 0.2; NaCl 0.1; $CaCO_3$ 3.0; Yeast extract 0.2; and agar-microbiologist 15.0; and sterilized at 121 °C for 15 minutes (Vincent, 1978). Sterilized congo red solution (1%) was added into the sterilized medium at 2.5 ml/L medium.

***Qualitative examination of paraquat effects on* Rhizobium**. This examination was based on the paper disc diffusion technique. Sterilized paper discs were put into a series concentration of paraquat solution (0 to 100 ppm), and were put on YMA medium, which was inoculated (surface plated) by *Rhizobium* sp. They were incubated at room temperature for 72 hours. Paraquat inhibition was measured after incubation time based on diameter of inhibition zone (clear zone) around the paper disc.

***Quantitative examination of paraquat effects on* Rhizobium**. This examination was based on the changes of population density of *Rhizobium* sp. in a synthetic medium containing paraquat. The bacteria were grown in YM broth medium added with paraquat at initial density around 10^6 cell/mL, and incubated on a shaker incubator (125 rpm) at room temperature for 48 – 72 hours. Several concentrations of paraquat were applied, namely 0 (control), 20, 40, and 100 ppm. Bacterial density was measured periodically using surface plating count method in YMA medium.

RESULTS AND DISCUSSION

In qualitative examination by paper disc diffusion technique, it was shown that paraquat caused inhibition to the growth of *Rhizobium* (Fig.1). Paraquat inhibited some strains of *Rhizobium*. The Rj-143 (*R. japonicum* strain 143) was inhibited at the highest level, and then followed by *Rhizobium* sp. strain G-69, G-182, Rj-96, KS and CC-1.1. *Rhizobium* sp. strain T-37 and QF were not inhibited by paraquat although the concentration was 100 ppm. (Fig. 1).

These results indicated that the effect of paraquat on microorganisms is species specific, which means that different mechanism might occur on each strain. As written in the report of Carr *et al.*, (1986), different toxicity mechanism were observed in different species. Generally accepted mechanism for paraquat toxicity is species specific and in some cases the growth inhibition by paraquat is caused by the reaction of the herbicide with unidentified cellular moieties or biochemical processes (Carr *et al.*, 1986).

Anderson (1978) reported different species of *Rhizobium* showed different tolerance to herbicides. Namely, *R. meliloti, R. trifolii* and *R. leguminosarum* were inhibited by some herbicides at 1000 ppm; but *R. lupini* and *R. japonicum* have been inhibited at 100 ppm. Katayama and Kuwatsuka (1992) also reported that the bacteria of *Escherichia coli* K-12 and *Pseudomonas* sp. TT01; the yeast of *Lipomyces starkeyi* resistant to 1000 ppm of paraquat. Additionally, *Aspergillus niger* and *Penicillium frequentans* were resistant to paraquat until 2000 ppm.

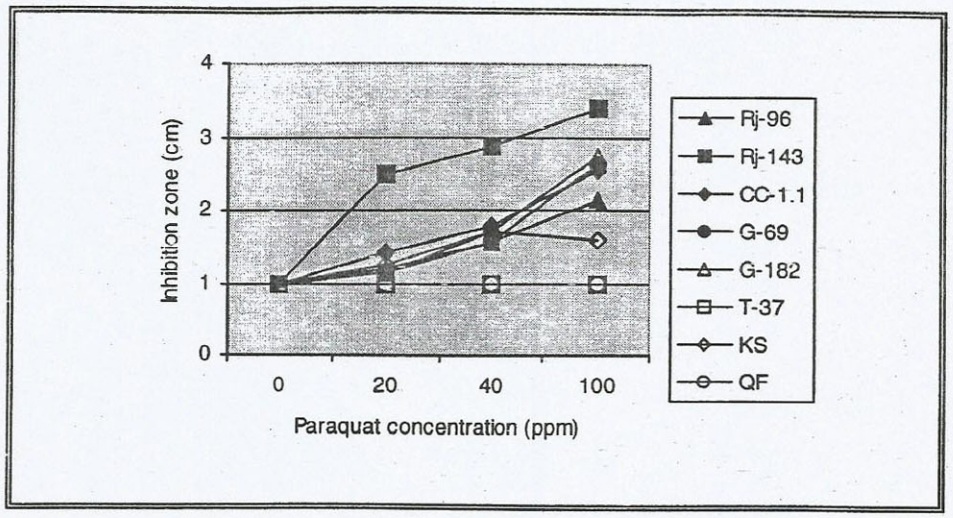

Figure 1. The increase of paraquat inhibition qualilatively on several strains of *Rhizobium*

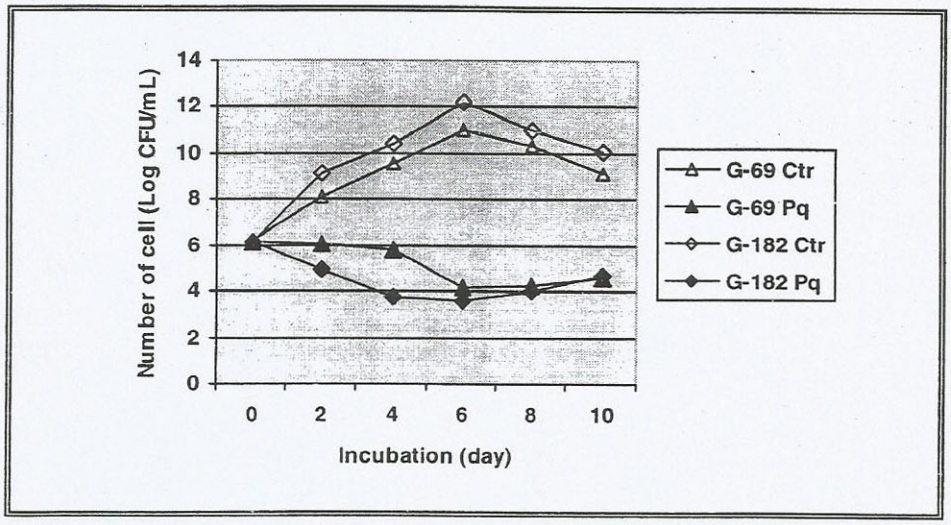

Figure 2. Effects of paraquat at 100 ppm on the growth of *Rhizobium* sp. strain G-69 and G-182.
Notes : blank symbols = control; black symbols = treated with paraquat

Table 1. Paraquat effect on the growth of *Rhizobium* sp. G-69 and G-182.

Rhizobium Strain	Paraquat Concentration	Cell number (Log CFU/mL)					
		Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
G-69	0 ppm	6.257 a	8.067 a	9.430 a	1.033 a	10.350 a	9.113 a
	100 ppm	6.117 b	6.040 b	5.883 b	4.003 b	4.270 b	4.673 b
G-182	0 ppm	6.130 a	9.140 a	10.393 a	2.233 a	11.050 a	10.083 a
	100 ppm	6.177 a	4.963 b	3.747 b	3.747 b	3.600 b	4.720 b

Notes : Significant differences between treatments were shown by different letters following the numbers in column or row at 5% significance level of T-test.

The resistance or sensitivity of microorganisms to paraquat is depending on their ability to neutralize paraquat toxicity. Paraquat auto-oxidation and photo-degradation produced some free radicals superoxides, i.e. H_2O_2, O_2^- and $^1O_2$ which can damage cell membrane and cell configuration, inhibit protein synthesis and cell division (Carr *et al.*, 1985; Hassan *et al.*, 1978). The tolerance and degradative ability to paraquat are associated with the integrity of cell wall (Carr *et al.*, 1986).

Paper disc diffusion technique was besed on diameter of inhibitory zone and can not distinguish bacteriostatic or bacteriocidal action of the substance (Reiner, 1977). To investigate more detail toxicity mechanism of paraquat to *Rhizobium* sp. a quantitative examination was required. Some strains of *Rhizobium*, namely *R. japonicum* strain 143, *Rhizobium* sp. strain G-69, G-182, and the isolate of KS were selected for quantitative examination in the effect of paraquat on *Rhizobium* growth. The results were shown in Fig. 2, Fig. 3 and Fig. 4.

Figure 2 shows that without paraquat addition, *Rhizobium* grew and increased their density from 10^6 to 10^{12} CFU/mL. It means that the bacteria used mannitol contained in the medium (Rao, 1994) as carbon source. Addition of paraquat at 100 ppm, slightly decreased cell density of *Rhizobium* sp. strain G-69 and G-182 from 10^6 to 10^4 CFU/mL at the early incubation, but then recovered to 10^5 CFU/mL until the end of incubation. Although the population was relatively stable, compared with control, there was significant decrease of cell number (Table 1). It was suggested that paraquat did not damage the cell of G-69 and G-182, but only inhibit the growth or cell division. At the end of incubation these strains have recovered and adapted to paraquat. These data were supported by Hassan *et al.* (1978), which show that paraquat inhibits protein synthesis and cell division. Carr *et al.* (1986) also reported that paraquat delayed lag-phase of *Lipomyces starkeyi,* but then this yeast enter in logarithmic phase and started to degrade the paraquat.

Different phenomena were observed in the cases of other isolates, namely *R. japonicum* strain 143, *Rhizobium* sp. strain KS. (Fig. 3 and Fig. 4). These strains showed similarity on their reaction to paraquat.

At 0 ppm paraquat, cell density increased from 10^6 CFU/mL to 10^8, or 10^9 CFU/mL (Fig. 3 and Fig. 4). These data were coincided with those in Fig 2, in which *Rhizobium* grew using mannitol as carbon source. These high level of population required high amount of carbon source. Therefore, after eight day-incubation period the population decreased due to the limiting carbon source.

Table 2. Paraquat effect on the growth of Rj-143.

Paraquat concentration	Cell number (Log CFU/mL)					
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
0 ppm	6.240 bc	8.560 a	8.175 a	7.720 a	7.240 a	6.000 b
20 ppm	6.850 a	5.475 b	8.395 a	7.180 b	7.210 a	7.435 a
40 ppm	6.595 ab	4.770 c	8.465 a	6.180 c	6.380 b	6.535 b
100 ppm	6.045 c	3.705 d	3.590 b	2.630 d	0.650 c	0.000 c

Notes: Significant differences between treatments were shown by different letters following the numbers in column or row at 5% significance level of DMRT (Duncan's Multiple Range Test).

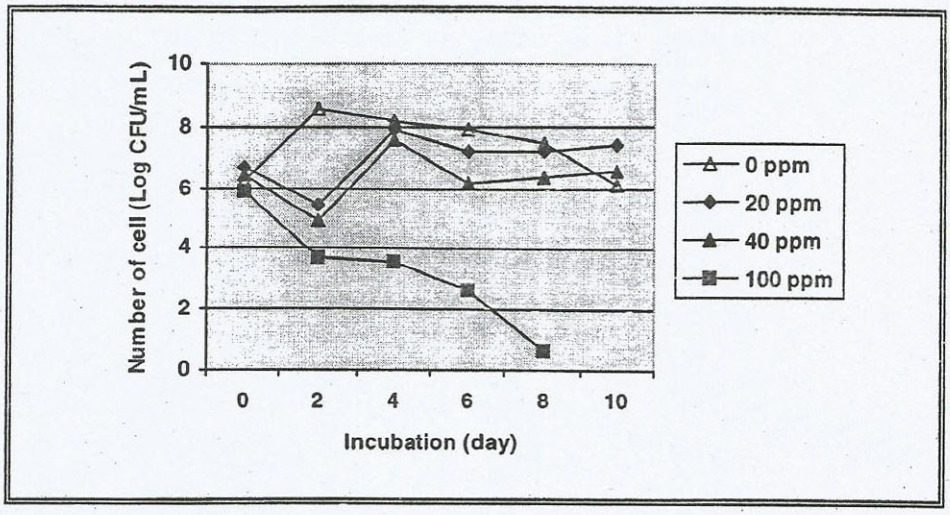

Figure 3. Influence of paraquat on the growth of *R. japonicum* strain 143.

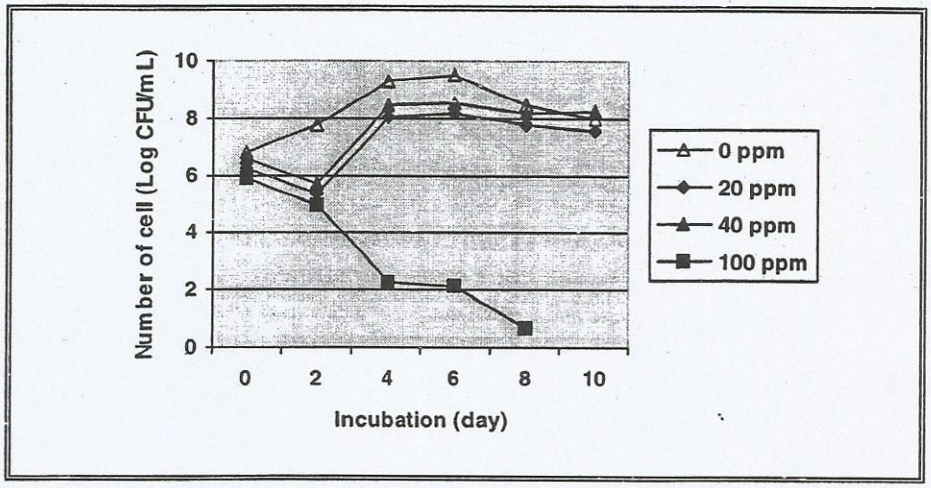

Figure 4. Influence of paraquat on the growth of *Rhizobium* sp. strain KS.

Table 3. Paraquat effect on the growth of KS.

Paraquat concentration	Cell number (Log CFU/mL) Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
0 ppm	6.385 a	7.790 a	9.300 a	9.580 a	8.355 a	8.020 a
20 ppm	6.535 a	5.620 b	8.065 b	8.240 c	7.820 a	7.605 a
40 ppm	7.195 a	5.690 b	8.630 b	8.555 b	8.180 a	8.320 a
100 ppm	6.025 a	4.955 b	2.110 d	2.110 d	0.720 b	0.000 b

Notes: Significant differences between treatments were shown by different letters following the numbers in column or row at 5% significance level of DMRT (Duncan's Multiple Range Test).

However, addition of paraquat at concentrations of 20 and 40 ppm, cell density decreased from 10^5 CFU/mL in early incubation. Then the population increased to the level of 10^6 or 10^8 CFU/mL (Fig. 3 and Fig. 4). The *R. japonicum* strain 143 and *Rhizobium* sp. strain KS showed similar growth curves (Fig. 3 and Fig. 4). The statistical data were shown in Table 2 and Table 3. It was suggested that these three strains required a couple of days for adaptation to paraquat. After successful adaptation they began to grow, although the maximal population were lower than those in medium without paraquat. These data were supported by Carr *et al.*, (1985) using *Lipomyces starkeyi*. Carr *et al.*, (1986) insisted that some microorganisms could synthesize a number of enzymes which can neutralize the paraquat toxicity caused by the produced free radical superoxydes. In some bacterial cells, paraquat detoxification may involved the O_2^- detoxification by the enzyme superoxide dismutase (SOD), giving the rise to H_2O_2, which in turn is removed by catalase (Carr *et al.*, 1986). Synthesis of SOD in *E. coli* and *L. starkeyi* was induced by O_2^-, and the catalase of *Bacillus subtilis* and *L. starkeyi* was induced by H_2O_2.

Different phenomena were observed when paraquat was added at 100 ppm, namely the cell density of *R. japonicum*-143 and *Rhizobium* sp. strain KS decreased to 10^1 CFU/mL or below (Fig. 3 and Fig. 4). Table 2 and Table 3 show statistical data of the paraquat effect to the population of these rhizobial strains. These data indicated that higher paraquat concentration caused higher deleterious effect, which lead to the death of the cell. The same phenomena were observed in the case of *Rhizobium* sp CC-1.1 which was isolatred from *Paraceriantes falcataria* (data not shown). Carr *et al.* (1986) and Hassan *et al.* (1978) reported that a possible mechanism for paraquat toxicity to organisms is that the free radical superoxides of paraquat damage cell membrane and cell configuration. Therefore, these three *Rhizobium* strains could not recover from the paraquat toxicity at 100 ppm.

The data in Fig. 3 and Fig. 4 indicated the possibility that different toxicity mechanism were occurred at different paraquat concentrations. It was suggested that at low concentration, paraquat did not caused cell damage and the bacteria were tolerant to the paraquat toxicity. However, toxicity of 100 ppm might be caused by bacteriocidal action of paraquat which gave irreversible cell damage and lead to the death of the cell. As mention by Reiner (1977), the same substance can show different toxic mechanisms to a particular population depend on various factors, such as its concentration.

Many studies showed that *Rhizobium* cell number influence the formation of root nodule in legumes. In laboratory experiment, cell density of 10^6 or 10^7 cell/mL is optimal for nodule formation (Roughly & Pulsford, 1982); but field experiment needs higher density, i.e. 10^8 or 10^9 cell/mL (Rao, 1994). Without root nodules and nitrogen fixation, growth of legume is not optimal and needs addition of high level of nitrogenous fertilizer.

This study show that paraquat decreased cell density of *Rhizobium* around two to five magnitude levels, depend on paraquat concentration and the rhizobial strain. These results are important due to role of *Rhizobium* in root nodule formation, nitrogen fixation and plant resistance to pathogens and herbivores (Hammerschmidt & Smith-Becker, 1999; Karban & Kue, 1999). Study concerning the influence of paraquat on *Rhizobium* and the relationship with root nodule formation in legume plants is required in the future.

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