Jurnal Perlindungan Tanaman Indonesia, Vol. 10, No. 2, 2004 : 87 - 96.

PARAQUAT TOXICITY ON ROOT NODULE FORMATION ON Macroptilium atropurpureum Urb. AND ITS CORELATION WITH POPULATION OF Rhizobium sp.

TOKSISITAS PARAKUAT TERHADAP BINTIL AKAR PADA TANAMAN SIRATRO (Macroptilium atropurpureum Urb.) DAN KAITANNYA DENGAN POPULASI Rhizobium sp.

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INTISARI

Penelitian ini dilakukan untuk mengetahui toksisitas parakuat terhadap pembentukan bintil akar oleh Rhizobium *sp. pada tanaman siratro (*Macroptilium atropurpureum*) sebagai tanaman indikator. Dua strain* Rhizobium, *yaitu* R. japonicum *143 (Rj-143) dan* Rhizobium *sp. CC-1.1 (CC-.1), dipilih untuk mewakili grup kedelai dan penutup tanah. Siratro ditumbuhkan dalam medium Thornton cair yang telah diinokulasi* Rhizobium *dan diperlakukan dengan paraquat pada beberapa konsentrasi. Selama masa pertumbuhan, diamati pembentukan bintil akar, dan populasi* Rhizobium *dalam medium. Kemudian pada akhir masa tanam dihitung aktivitas nitrogenase bintil akar dengan metode ARA (Acethylene Reduction Analysis).*

Hasil pengamatan menunjukkan bahwa pada tanaman yang diinokulasi Rhizobium *tanpa penambahan parakuat, bintil akar terbentuk pada minggu keempat. Penambahan parakuat menurunkan jumlah bintil atau bahkan pada konsentrasi tinggi menyebabkan kegagalan terbentuknya bintil. Gejala toksisitas parakuat pada siratro yang berupa khlorosis, kerdil, keringnya jaringan atau kematian tanaman; mulai nampak pada minggu kedua. Pada konsentrasi 20 ppm populasi* Rhizobium *relatif konstan, tetapi di atas 40ppm herbisida ini menyebabkan penurunan populasi dari 10^6 menjadi 10^1 CFU/mL. Nampak bahwa tinggi-rendahnya toksisitas tergantung pada konsentrasi parakuat yang ditambahkan dan strain bakteri yang digunakan, dimana Rj-143 lebih toleran dibandingkan CC-1.1.*

Hasil diatas menunjukkan bahwa parakuat telah mengganggu pertumbuhan siratro sejak sebelum terbentuknya bintil, dan pada saat bersamaan Rhizobium *juga menurun populasinya. Akibatnya, pembentukan bintil akar terhambat, atau bahkan dalam beberapa perlakuan bintil akar tidak terbentuk. Hal ini merupakan peringatan bahwa kita harus berhati-hati mengaplikasikan pestisida, khususnya herbisida dengan bahan aktif parakuat.*

Kata kunci : Bintil akar, Parakuat, *Rhizobium*, Siratro.

ABSTRACT

This study was designed to investigate the paraquat toxicity toward root nodulation by *Rhizobium* on *Macroptilium atropurpureum* as an indicator plant. The legume was grown in Thornton medium treated with several concentrations of paraquat and inoculated with *R. japonicum*

143 (Rj-143) or *Rhizobium* sp. C-1.1. These bacteria represent cross-inoculation groups of soybean and cover-crop legumes, respectively. Nodule formation and *Rhizobium* population were measured periodically. At the end of planting time, nitrogenase activity of the nodules was analysis based on ARA (Acethylene Reduction Analysis) method.

The results showed that nodules in plants inoculated with *Rhizobium* without addition of paraquat, were formed within four weeks. There was no nodulation when paraquat was added. Paraquat was toxic to the plant, causing chlorosis, stunting, drying of the plant tissues, and death. The symptoms were detected at the second week after planting time. Paraquat also decreased Rhizobium population from 10^6 to 10^2 or 10^1 CFU/mL at 40 and 100 ppm, respectively. These results depicted that paraquat disturbed the plant before nodulation, and at the same time *Rhizobium* population decreased until below minimal population required for nodulation. Therefore, the process of nodulation was disturbed, and in some treatments there was no nodulation. It was concluded that paraquat was toxic to both plant and the Rhizobium, which cause nodulation failure.

Key words : *Macroptilium atropurpureum,* Paraquat, *Rhizobium,* and Root nodules.

INTRODUCTION

Paraquat is an active agent of several herbicides widely used in agricultural lands including peat land and rain fed agricultural system. This substance is highly toxic to organisms, nonbiodegradable, and accumulated in natural environments, causing negative impacts to nontarget organisms. Routine application of paraquat may increase the concentration of paraquat residue in soil which in turn will decrease soil fertility and productivity. Paraquat inhibited the growth of microorganisms and decreased microbial biomass (Katayama & Kuwatsuka, 1992), or changed the dynamics of microbial population (Margino *et al.,* 2000), nitrifier bacteria (Setyaningsih *et al.*, 2001), and *Azotobacter* sp. in soil (Anderson, 1978).

Negative impacts of paraquat on the growth of corn and rice planted in peat soil, were reported by Martani *et al.* (2000; 2002). Paraquat also inhibited soybean growth of and decreased the number of root nodules, and the yield (Martani *et al.,* 2001). It was suggested that paraquat is toxic to *Rhizobium* sp., which has an important role in supplying nitrogen to the plants from root nodule.In efficient fixation, *Rhizobium* supply enough nitrogen for the plant, and minimize the requirement of fertilizer.

Although there is an indication that paraquat affected *Rhizobium,* its toxicity mechanism is not clear yet, especially in correlation with nitrogen fixation (Martani *et al.*, 2001). Is paraquat cause growth inhibition to *Rhizobium*; disturb the process of nodulation; decrease the activity of nitrogenase enzyme(s); or paraquat affect all of the procceses in symbiotic mutualism between *Rhizobium* and the legume for nodulation and nitrogen fixation?

Information about mechanism of paraquat toxicity to *Rhizobium* is important since the role of these bacteria is not only in nitrogen fixation but also in crop resistence to pathogen and herbivores by producing phytoallexin (Hammerschmidt & Smith-Becker, 1999; Karban & Kue, 1999). Therefore, the negative impact of paraquat to *Rhizobium,* nodule formation, and nitrogen fixation, in turn will cause growth inhibition, and decrease the yield. In this study these problems were elucidated.

Macroptilium atropurpureum Urb. (Siratro[Ind]) was used as indicator legume plant for nodulation effectivity of *Rhizobium.* This plant was chosen due its widely mutualistic specification between *M. atropurpureum* and *Rhizobium* (Allen & Allen, 1981), and short

planting time for nodule formation.

MATERIALS AND METHODS

Paraquat herbicide. *Gramoxon*$^{(R)}$ (Zeneca Ltd. Co.); active agent 200 mg paraquat ion/L as paraquat source.

Macrotilium atropurpureum. This legume was used as legume indicator for nodule formation. The seed germinated for 4 – 5 days on a wet sterile filter paper before used as indicator plant.

Rhizobium sp. strains. Two strains of *Rhizobium*, *R. japonicum* 143 and *Rhizobium* sp. CC-1.1 were chosen and used for this experiment based on their sensitivity to paraquat (Martani, 2002).. They were isolated from different cross-inoculation group, namely: Rj-143 from soybean root nodules represents *Rhizobium* compatible with soybean group; while CC-1.1, from *Paraceriantes falcataria* (former name *Albizia falcataria*), and is compatible with cover crop legumes.

Before the inoculants were used for nodulation experiment, they were precultured in a Yeast Extract Mannitol Agar (YMA) medium broth on shaker incubator (125 rpm) for 2 – 3 days.

Nodulation experiment using M. atropurpureum (Allen & Allen, 1981).. The young plants were inserted into modified test tube containing 50 mL of Thornton Broth Medium (Vincent, 1982) inoculated with *Rhizobium* sp. at initial population 10^6 cell/mL and added with paraquat at 0 to 100 ppm. They were incubated in direct sunshine for eight weeks.

To avoid the effect of sunshine to *Rhizobium*, the tube was covered with black plastic. New medium was added into the tube periodically to keep volume of medium constant at around 50 mL.For anticipation of the nitrogen deficiency on uninoculated plants, 70 ppm of KNO_3 was added into the control tubes (without *Rhizobium* inoculation) (Rao, 1994).

Population of *Rhizobium* were measured periodically using plate count method on YMA medium containing congo-red 0.1% (Rao, 1994). At the end of incubation (8 weeks), the number and weight of root nodules was counted. Nitrogenase activity of the root nodules were measured by using Gas Chromatography based on Acethylene Reduction Analysis (ARA) method.

RESULTS AND DISCUSSIONS

The ability of these strains to form root nodule in Thornton medium containing several concentration of paraquat were shown in Table 1. Based on visual examination by formation of leghaemoglobin after crushing the nodules, all of the nodules were identified as effective, which means that they were able to fix nitrogen.

Table 1 showed that in control I (no paraquat addition), *M. atropurpureum* was able to grow, but failed to form nodule. About 4-5 weeks, the leaves turned yellowish (chlorosis) and the root grew abnormally long. Addition of nitrogen into the medium (Control II) caused better growth of *M. atropurpureum* and no chlorosis was detected, but still there was no formation of root nodules. It means that addition of KNO_3 was able to fullfill the nitrogen requirement for the plants (Vincent, 1982).

Those data showed that *Rhizobium* is required in root nodule formation; as the nodule is a result of mutualistic symbiosis between legumes and compatible *Rhizobium* species (Vincent, 1982). The abnormal growth of the plant (chlorosis and very long root) represents nitrogen deficiency in medium. Due to the absence of nitrogen source in medium for plant growth, root nodules are required to supply nitrogen. Paraquat toxicity to these plants was detected at 1 - 2nd week. Even at 20 ppm paraquat caused chlorosis on leaves and

then the plant died.

This herbicide also decreased the number, size, and dry weight of root nodules. In medium with paraquat, at weeks 4th the Rj-143 formed two root nodules with dry weight of 0.016 g/nodule (Table 1). However, addition of paraquat decreased the nodule size and dry weight significantly, namely, at 20 ppm paraquat the numbers were 11, but their dry weight decreased to 0.008 g/nodule. At 40 and 100 ppm, the Rj-143 failed to form nodule, the plant turned yellow, and then died (Figure 1).

Different reaction was shown by CC-1.1 in medium without paraquat, in which after four weeks the plant formed 32 nodules with average weight 0.015 g/nodule. No nodule was formed in paraquat added medium, even at 20 ppm (Table 1). The leaves showed chlorosis, the plant stunted, dry, and then died (Figure 2). These data indicated that CC-1.1 was more sensitive to paraquat than Rj-143.

Growth inhibition, disturbing and dying of *M. atropurpureum* indicated that paraquat was toxic to this plant, including to the formation of root nodule. Martani *et al.*, (2001) reported the inhibition of growth and yield decrease of soybean planted in peat soil treated with paraquat herbicide. Although paraquat toxicity has also been reported else where (Martani *et al.*, 2000; 2002), the detail mechanism has not been elucidated yet.

It is widely known that paraquat toxicity to weeds was due to its free radical substances that disturb photosynthesis (Carr *et al.*, 1985; Hassan *et al.*, 1978). As a contact herbicide, paraquat only toxic to the exposed plant organels and its translocation in plant tissues is very slow. In broth medium paraquat can be absorbed by the root and were translocated into all of plant tissues, included the leaves (Soejono, 1986). The above theory was proven in this study, and may explain the mechanism of paraquat toxicity to nontarget organisms/crops.

Table 1. Root nodule formation in *M. atropurpureum* after 8 weeks.

Treatment / Nodule measurements	Paraquat concentration (ppm)			
	0	20	40	100
1. Control I [1)]				
Number of nodules	0	0	0	0
Total weight of nodules (g)	--	--	--	--
Average weight of each nodule (g)	--	--	--	--
2. Control II [2)]				
Number of nodules	0	0	0	0
Total weight of nodules (g)	--	--	--	--
Average weight of each nodule (g)	--	--	--	--
3. *R. japonicum* 143 (Rj-143)				
Number of nodules	2 [3)]	11	0	0
Total weight of nodules (g)	0.032	0.087	--	--
Average weight of each nodule (g)	0.016	0.008	--	--
4. *Rhizobium* sp. CC-1.1 (CC-1.1)				
Number of nodules	32 [3)]	0	0	0
Total weight of nodules (g)	0.476	--	--	--
Average weight of each nodule (g)	0.015	--	--	--

Notes: [1)] Without Rhizobium inoculation, medium without KNO_3 70 ppm
[2)] Without Rhizobium inoculation, medium was added with KNO_3 70 ppm
[3)] The nodule started to form at week 4.

Table 2. *M. atropurpureum* conditions at week 8.

Treatment / Growth Parameter	Paraquat Concentration (ppm)			
	0	20	40	100
1. Control I [1]				
Plant height (cm)	-- [3]	-- [3]	-- [3]	-- [3]
Dry weight plant (g)	-- [3]	-- [3]	-- [3]	-- [3]
Dry weight root (g) [2]	-- [3]	-- [3]	-- [3]	-- [3]
2. Control II [4]				
Plant height (cm)	7.3	-- [3]	-- [3]	-- [3]
Dry weight plant (g)	0.161	-- [3]	-- [3]	-- [3]
Dry weight root (g) [2]	0.097	-- [3]	-- [3]	-- [3]
3. Rj-143				
Plant height (cm)	8.5	10.3	8.0	5.0
Dry weight plant (g)	0.092	0.072	0.003	0.003
Dry weight root (g) [2]	0.110	0.174	0.007	0.004
3. CC-1.1				
Plant height (cm)	38,0	7,5	5,0	5,0
Dry weight plant (g)	0,306	0,016	0,019	0,002
Dry weight root (g) [2]	0,832	0,024	0,018	0,011

Notes : [1] Without *Rhizobium* inoculation, the medium without KNO_3
[2] Excluding root nodule
[3] The plant turned yellowish (chlorosis) before 8 weeks incubation period
[4] Without *Rhizobium* inoculation, medium was added with KNO_3 70 ppm

Paraquat caused chlorosis, growth inhibition, drying, and dying of the *M. atropurpureum* (Figure 1 and Figure 2). With higher paraquat concentration in the medium, higher toxicity of this chemical was detected. The growth inhibition, in turn will affect root nodule formation and also its nitrogen fixation. A healthy plant excretes high enough homoserine and lectine substances required by *Rhizobium* to start root infection for nodule formation (Graham & Halliday, 1977). Abnormal plant growth affects synthesis and excretion of the substances, which inhibit rhizobial infection. Here, abnormal growth of *M. atropurpureum* caused by paraquat was detected before, during and after root the nodule formation.

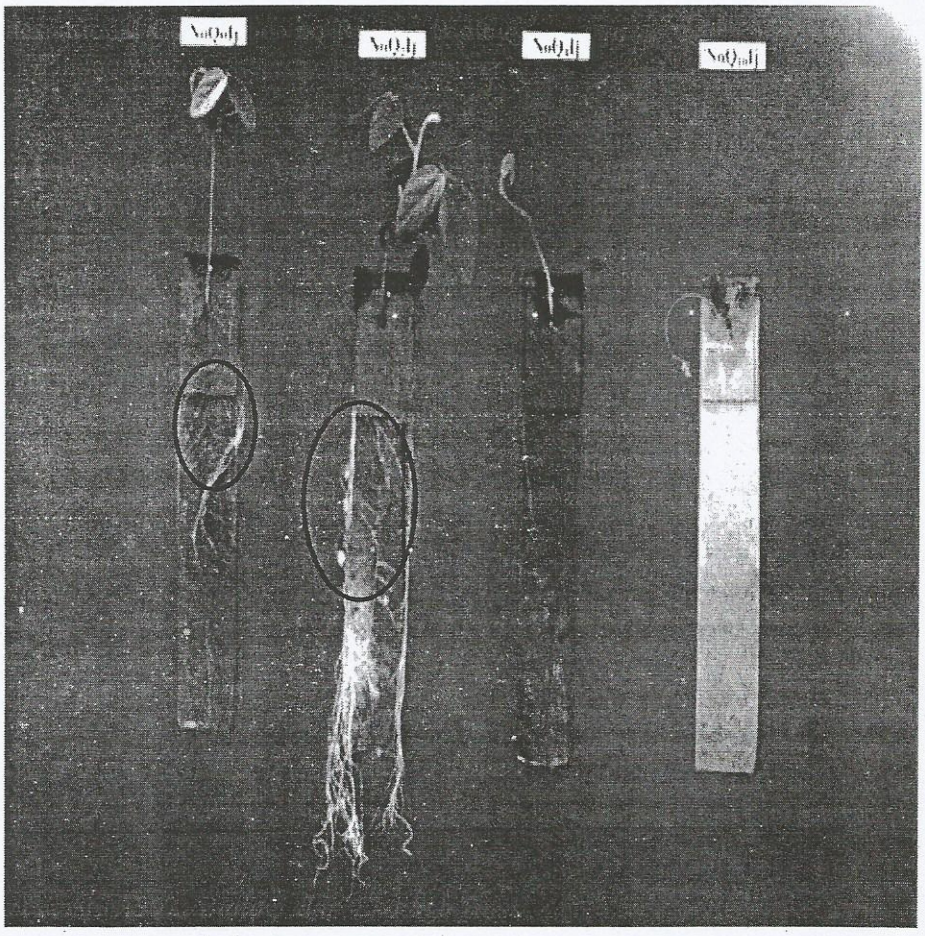
Nodule formation requires some steps, namely, the first is the compatibility between Rhizobial with legume species. Compatible symbionts resulted in the bacterial root infection; which followed by formation of infection thread in the root tissues, formation of bacteroid tissues, and the root nodule itself. Paraquat toxicity to the plant caused the plant die; which may occur before, during or after nodulation process. These will affect one, or all of the above steps, which in turn may cause the failure of nodule formation, decrease the number and weight of nodule, or also the nitrogenase activity of nodules.

In this study, it is proved that the failure of nodule formation was due to paraquat toxicity toward the plant itself. Nodulation was started after the legume was planted 4 weeks; and the toxicity symptom (chlorosis) was shown at first or second week depend on paraquat concentration.

Another possibility is that paraquat decrease *Rhizobium* population until a level

below minimal density required for nodule formation (Roughley & Pulford, 1982). Our preliminary study showed that paraquat was toxic and decreased population of *Rhizobium* grown in YM broth medium (Martani, 2002). If the same phenomenon occurred also in Thornton medium, it should be responsible for the failure of nodule formation in *M. atropurpureum*. To examine this possible explanation, *Rhizobium* population was also measured periodically.

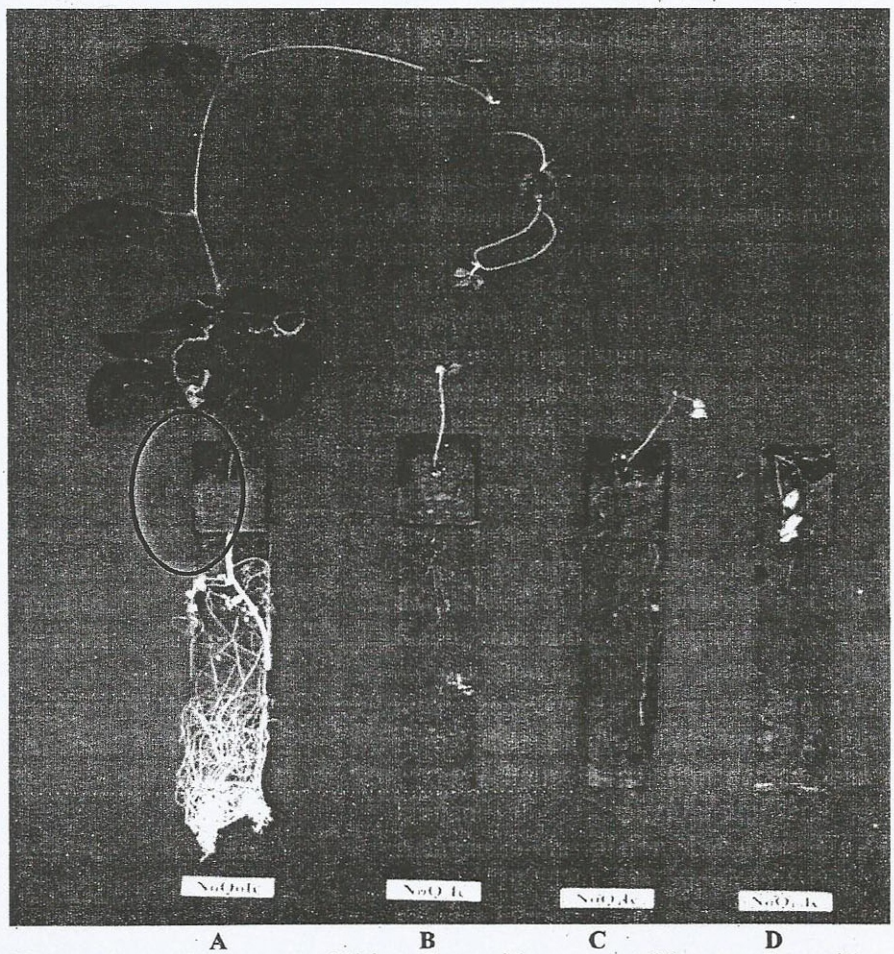
Figure 1. *M. atropurpureum* condition after 8 weeks in broth medium inoculated with *R. japonicum* 143.



Notes : A = no paraquat addition, B = with paraquat 20 ppm; C = with paraquat 40 ppm; D = with paraquat 100 ppm.

Circles show the root nodules.

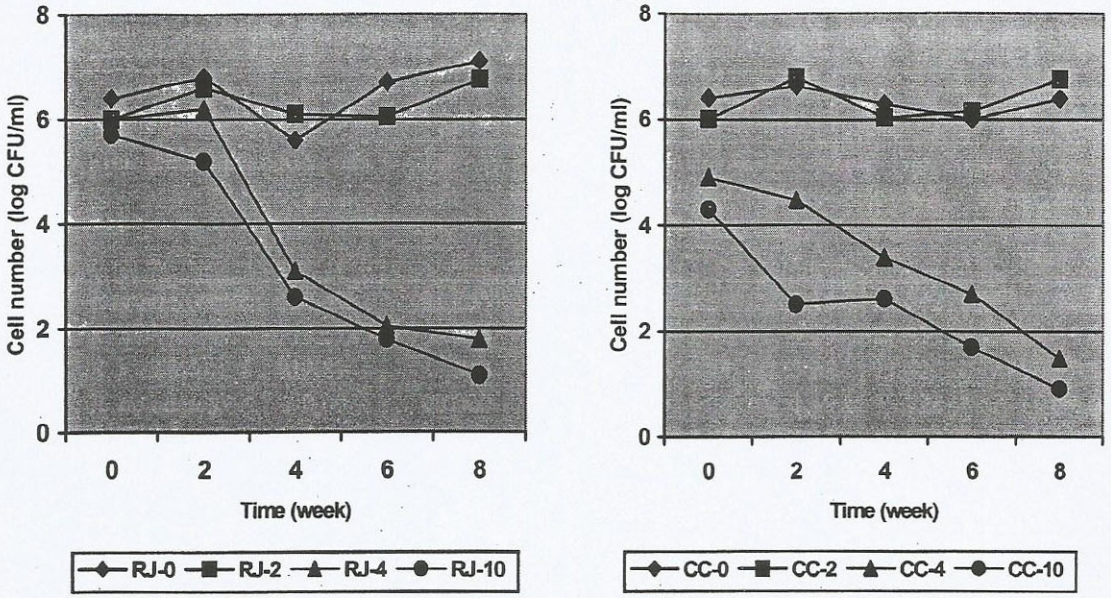
Figure 2. *M. atropurpureum* condition after 8 weeks in Thronton medium inoculated with *Rhizobium* sp. CC-1.1



A B C D

Notes : A = no paraquat addition, B = with paraquat 20 ppm; C = with paraquat 40 ppm; D = with paraquat 100 ppm.
Circle show the root nodules.

Figure 3 shows the Rj-143 and CC-1.1 population in Thornton medium with or without paraquat addition. In medium without paraquat, *Rhizobium* population is relatively constant at 10^6 – 10^7 CFU/mL (Figure 4). Therefore, the plant was able to form nodule (Tabel 1). Addition of 20 ppm paraquat was not significantly affected Rj-143 and CC-1.1 population, and nodule formation (Table 1). However, paraquat at 40 and 100 ppm decreased population significantly. At week 4 the population decreased to 10^3 or 10^2 CFU/mL, and continuously decreased until 10^1 CFU/mL at week 8. At high paraquat concentrations, nodule was not formed (Table 1). Martani (2002) reported that Rj-143 population in Yeast Extract Mannitol broth medium with 100 ppm paraquat decreased from 10^6 to 10^1 cell/mL within 8 days. It was suggested that the failure of nodule formation in *M. atropurpureum* was caused by the *Rhizobium* population was under minimal requirement density, that is 10^6 – 10^7 cell /mL (Roughley & Pulsford, 1982).



A B

Figure 3. The effect of paraquat on the growth of *Rhizobium japonicum* strain 143 (A) and *Rhizobium* sp. strain CC-1.1 (B) in Thronton medium
RJ-0 and CC-0 = without paraquat; RJ-2 and CC-2 = paraquat 20ppm;
Rj-4 and CC-4 = paraquat 40ppm; Rj-10 and CC-10 = paraquat 100ppm.

It is well known that paraquat superoxide damage cell membrane, cell configuration, and also inhibit protein synthesis and cell division (Carr *et al.*, 1985; Hassan & Fridovich 1978). Abnormal cell condition is responsible to the failure of CC-1.1 to form root nodule in medium with paraquat. The process for nodulation is affected not only by the *Rhizobium* density, but also by its survival and vigor (Vidor, 1981). Pasaribu *et al.*, (1989) also insisted that *Rhizobium* population and vigor is responsible to the success or failure of *Rhizobium* inoculation in soil. These phenomena were correlated with optimal cell metabolism, which in turn will affect formation of nodule and nitrogenase activity.

Some discrepancies could be observed between laboratory and field experiments, in which toxicity in laboratory scale is usually higher than in the soil. In case of synthetic medium, bacterial cell and root plant were directly exposed to paraquat; but in soil, some molecules of paraquat will be adsorbed by clay or organic materials, resulted in inactivity of this herbicide. Since paraquat is a contact herbicide, its toxicity in broth medium was higher than in soil.

The results suggested that the possibility of negative impacts of paraquat to *Rhizobium,* the one from thousands microorganisms important in biological processes in natural environments should be carefully considered. Microbial population disturbance may decrease soil quality and productivity. The impacts of pesticides on environments and non-target organisms are widely studied and proved (Anderson, 1978; Alexander, 1993), but until presently it is still happening.

CONCLUSIONS

1. Paraquat affected the formation of root nodules in *Macroptilium atropurpureum* by *Rhizobium japonicum* 143 and *Rhizobium* sp. CC-1.1. Higher paraquat concentration caused higher effect.
2. Paraquat caused chlorosis, stunted, drying and then death of the plant, and decreased *Rhizobium* population and its survival.
3. There was a tendency that the toxicity mechanisms to the root nodule at low and higher concentration was different.

ACKNOWLEDGEMENTS

This research could be conducted due to fund from Basic Research Project 2003 via The Research Institute of Gadjah Mada University. We thanks to Prof. Dr. Bostang Rajagukguk of Department of Soil Science, Fac. of Agriculture for valuable suggestion. Also to Kurniawan Wibowo SP. for his deligent work in this research.

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