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INFLUENCE OF PARAQUAT HERBICIDE ON SOIL BACTERIA, *Rhizobium* sp.
(Pengaruh Paraquat terhadap Bakteri Tanah, Rhizobium sp.)

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Abstrak

*Pencemaran pestisida merupakan salah satu masalah lingkungan yang menyebabkan gangguan terhadap organisme tanah. Paraquat adalah bahan aktif beberapa jenis herbisida yang banyak diaplikasikan di lahan gambut dan lahan pertanian tadah hujan. Studi ini dilakukan untuk mengetahui pengaruh herbisida paraquat terhadap bakteri Rhizobium sp. Tiga puluh lima strain Rhizobium sp. telah diuji dengan menggunakan teknik difusi cakram kertas (**paper disc**). Sebagian strain adalah hasil isolasi dari tanah, bintil akar tanaman leguminosa dan inokulum leguminosa (Legin – Legume Inoculum). Enam strain lain adalah bakteri Rhizobium japonicum. Hasil penelitian menunjukkan bahwa paraquat memiliki daya hambat terhadap bakteri Rhizobium sp.. Sebanyak 17,14% (enam strain) dari seluruh strain yang diuji, tidak mengalami penghambatan sampai konsentrasi paraquat 400 ppm. Bakteri ini memiliki prospek bagus untuk digunakan sebagai inokulum rhizobium terutama di lahan pertanian yang telah tercemari herbisida, khususnya yang mempunyai bahan aktif paraquat. Sebagian besar strain yang digunakan (82,86%) terhambat oleh 20 ppm paraquat, dan daya hambat tersebut makin besar seiring dengan meningkatnya konsentrasi paraquat. Mengingat makin meluasnya pemakaian herbisida berbahan aktif paraquat di Indonesia dan peran Rhizobium dalam fiksasi nitrogen, hasil penelitian ini memiliki arti penting, terutama bagi petani agar berhati-hati dalam pemakaian pestisida.*

Key words: Paraquat, Rhizobium sp.

Abstract

Pesticides may cause environmental pollution which lead to disturbance of soil biota. Paraquat is an active agent of herbicides usually used in peat land and rainfed agriculture. This research was conducted to examine the influence of paraquat on symbiotic nitrogen fixing bacteria, Rhizobium sp. Thirty five strains of the genus Rhizobium were examined in this research. Some of them were isolated from soils, legume root nodules, and Legume-Inoculum. Six strains of Rhizobium japonicum were also used in this research. Inhibiti on effect was based on paper disc agar diffusion technique with various concentrations of paraquat (Gramoxone (R)). The results showed that six strains of Rhizobium sp. (17.14%) were resistant to paraquat up to 400 ppm (w/w). These strains have a good prospect to be applied as Rhizobial-Inoculum especially in agricultural lands usually treated with paraquat. However,

most strains of Rhizobium sp. (82.86%) were inhibited by paraquat, and higher paraquat concentration caused higher degree of inhibition. Due to widely applied paraquat herbicides in Indonesia and the role of Rhizobium sp. in nitrogen fixation, these results were important, especially for farmers, in pesticide application.

Key words: Paraquat, Rhizobium sp.

I. INTRODUCTION

Except in organic farming, agricultural extensification and intensification increase the application of fertilizers and pesticides, which in turn will lead to environmental pollution. Pesticide residues may decrease soil and water quality, soil productivity, and change population dynamics of soil microorganisms (Anderson, 1978; Margino *et al.*, 2000; Martani *et al.*, 2000a & b). On the other hand, it is widely known that many soil microorganisms play an important role in bioremediation, nutrient cycle, and soil health. Disturbances of microbial population may cause the decrease of environmental quality and soil productivity.

This study was conducted to investigate the effects of paraquat on *Rhizobium* sp., a soil bacteria which play an important role in nutrient cycle through nitrogen fixation process. Many kinds of herbicides were used to inhibit the growth of weeds in peat land and also in rainfed agriculture. The widely used herbicides are Gramoxone and Paracol, in which Paraquat is their active agent.

Paraquat herbicide persists in soil due to its adsorption on clay mineral and organic substances (Anonymous, 1984). Although there are microorganisms which are resistant to paraquat until 1000 ppm (Katayama & Kuwatsuka, 1992), some studies have shown that paraquat influenced the growth of soil microorganisms (Anderson, 1978; Katayama & Kuwatsuka, 1992; Margino *et al.*, 2000; Setyaningsih *et al.*, 2001). It has been reported that 20 ppm of paraquat in soil changed the population dynamics of peat soil bacteria and fungi (Margino *et al.*, 2000) or nitrifying bacteria in some types of soils (Setyaningsih *et al.*, 2001). *Azotobacter* sp. is also affected by pesticides (Anderson, 1978). It was suggested that the change in number and dynamics of microorganisms, especially which are important in soil fertility, may influence the growth of crop. Martani *et al.* (2000 & 2001) showed that in greenhouse experiments, paraquat application in peat soil inhibited the vegetative growth and decreased the yield of soybean and corn. It does not clear yet, whether it was due to direct or indirect effect through the action on soil microorganisms. However, in her unpublished work, Martani noted that in liquid medium, addition of paraquat caused chlorosis, wilt and growth inhibition of *Macroptilium atropurpureum* Urb.

Rhizobium sp. is important not only in nitrogen fixation symbiotically with legumes, but also in plant resistance to plant pathogens (Hammerschmidt & Smith-Becker, 1999). This ability is due to the synthesis of salisylic acid which is one of the phyto-allexins substances which are important in plant resistance to pathogen and herbivores (Hammerschmidt & Smith-Becker, 1999; Karban & Kue, 1999). Therefore, it was suggested that disturbance of paraquat on *Rhizobium* sp. or *Bradyrhizobium* sp. might also be responsible the growth inhibition of soybean in peat soil treated with paraquat (Martani *et al.*, 2001).

This research was conducted to investigate the influence of paraquat on *Rhizobium* sp. Examination was done using paper disc agar diffusion technique. It was hoped that the results could be used as a basic information concerning the paraquat toxicity on non-target (micro-)organisms.

II. MATERIAL 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.

Isolation of Rhizobium sp. To obtain *Rhizobium* sp., several kinds of source were used in this study. One of them was Kalimantan peat soil which has been used in a greenhouse experiment. This peat soil has been treated with paraquat and planted with soybean (Martani *et al.*, 2001). Another source of isolates was a Legume-Inoculum. Isolation was done using surface plating method on Yeast-extract Mannitol Agar (YMA) medium added with Congo-red (Vincent, 1978). Clear-white and gummy colonies were characteristics of the genus *Rhizobium* (Vincent, 1978; Date, 1978). The *Rhizobium* suggested colonies were transferred and kept on YMA slants.

The cultures of Rhizobium sp. Other strains of *Rhizobium* were obtained from Microbial Culture Collection, Indonesian Institute for Sciences-LIPI, Bogor. Some other strains were received from Prana, Research & Development Centre for Biotechnology, Indonesian Institute for Sciences-LIPI, Bogor. All of these cultures were chosen based on the difference in source of each isolate. Several strains of *Rhizobium japonicum* were also used in this research.

Examination of paraquat effects. The effects of paraquat was examined by using paper disc agar diffusion technique (Yutono *et al.*, 1980). Each strain of *Rhizobium* sp. was grown by surface plating method in YMA medium. Before incubation, sterilized paper discs (F 10 mm) containing a series of paraquat concentrations were put aseptically on the medium. Paraquat concentrations used were 0, 20, 40, 100, 200 and 400 ppm. They were incubated at room temperature for 48 - 72 hours. Measurement of inhibition effect was based on the diameter of inhibition zone around the paper disc.

III. RESULTS AND DISCUSSION

A. *Rhizobium* sp. isolations and cultures.

Legume-Inoculum has been widely used in Indonesia on soybean crops since more than 10 years ago, especially in transmigration areas. It was chosen as source of isolate due to its high concentration of *Rhizobium* sp. Another source of isolate was peat soil that has been treated with paraquat. Martani *et al.* (2001) conducted a greenhouse research concerning the effect of paraquat on the growth of soybean in peat soil. After harvesting, the peat soil was taken and used as source of *Rhizobium* isolates.

Using surface plating method, an isolate was obtained from Legume - Inoculum. In addition, four strains were isolated from peat soil treated with paraquat. Another six strains of *Rhizobium japonicum* and 24 strains of *Rhizobium* sp. were obtained from Culture Collection Institutes in Bogor. The details of sources and code of each isolate shown in Table 1.

The *Rhizobium* sp. (Table 1) were grown in YMA slant. Almost all cultures were fast-grower as evident from their rapid growth and the size of the colony (Vincent, 1982). Some of these cultures were slow-growing *Rhizobium* sp., i.e. the strains G-183, GSL-48, and PF-20 (Table 4 and 5). However, although some of the cultures were slow growers, all cultures were examined for the inhibition effect of paraquat, because sometimes slow growers are still effective in nitrogen fixation (Vincent, 1978). To examine paraquat influence, the Rhizobium were pre-cultured in YMA medium for 24 - 48 hours.

Table 2 shows that several isolates of the *Rhizobium* sp., i.e. Leg and KS, were inhibited by paraquat, although the concentration was only 20 ppm. Inhibition zone was wider with the increase of paraquat concentration. Strain KS was isolated from paraquat untreated sapric peat soil that was planted with soybean (Martani *et al.*, 2001). Martani *et al.* (2001) showed that paraquat inhibited the soybean

growth and the number of root nodules. Another study reported that the growth of soil microorganisms important in nitrogen biotransformations were influenced by herbicides (Anderson, 1978), including paraquat (Margino *et al.*, 2000; Setyaningsih *et al.*, 2001).

Paraquat toxicity on non-target organisms is due to the production of free radical (superoxide substances) during auto-oxidation and photo-degradation of paraquat (Anderson, 1978; Glonn *et al.*, 1982). These substances of H_2O_2, O_2^-, OH^- and O_2 could damage cell membrane and influence the photosynthesis activity (Glonn *et al.*, 1982),.

Table. 1. The Cultures of *Rhizobium* sp.

Source of cultures	Number of strain	Code	Notes
I. Isolation	1	Leg	Isolated from Legume-Inoculum
	4	KS, KQH, KQF and QF	Isolated from Kalimantan peat soil
II. IIS-LIPI, Bogor [1)]	6	*R. japonicum*	*Rhizobium japonicum*
	10	G	Isolated from Soybean planted in peat soil
	2	R	Isolated from Soybean var. Ringgit
	2	T	Isolated from Soybean var. Taichung
	2	GSL	Isolated from Soybean var. Genjah Slawi
III. IIS-Biotechnology, Bogor [2)]	8	CC, KḂ and PF	Isolated from root nodules of *Paraccerianthes falcataria*
Total strain	**35**		

Note: [1)] Anonymous, 1996. [2)] Personal collection

Table 2. Inhibition of Paraquat on the Isolates of *Rhizobium* sp.

Rhizobium sp. strain	Diameter (cm) inhibition zone in some levels of paraquat concentrations (ppm)				
	0	20	40	100	400
Leg	0	1,158	1,383	1,808	[1)]
KS	0	1,258	1,725	2,542	[1)]
KQH	0	0	0	0	0
KQF	0	0	0	0	0
QF	0	0	0	0	0

Note : [1)] Not examined

Leg = isolated from Legume-Inoculum; KS = isolated from untreated paraquat sapric peat soil; KQH = isolated from paraquat and lime treated hemic peat soil, KQF = isolated from paraquat and lime treated fibric peat soil; QF = isolated from paraquat treated fibric peat soil.

Rhizobium sp. strain KQH, KQF and QF were not inhibited by paraquat at least up to 400 ppm (Table 2). The resistance was due to natural adaptation, because of these strains were isolated from paraquat treated peat soil planted with soybean. These results showed that the sources of rhizobial isolates influenced characters of the strain, especially its response to paraquat. Namely, *Rhizobium* strains which were isolated from paraquat polluted soil would have higher resistance to paraquat than those from non-polluted soil.

Rhizobial inoculation to increase nitrogen fixation in soybean is required especially in new agricultural lands or lands which never been planted with soybean (Date, 1982; Yutono, 1985). The paraquat resistant *Rhizobium* strains have a good prospect to be used as Rhizobial inoculum applied to agricultural lands which were repeatedly sprayed with (paraquat) herbicide. Survival of these strains in those lands are likely higher than the non-resistant strains. Paraquat persisted in soil, especially in soil with low pH and high concentration of organic substances and/or clay minerals (Anonymous, 1984; Alexander, 1994; Margino *et al.*, 2000).

The growth of six strains of *R. japonicum* was inhibited by paraquat (Table 3). The inhibition zone was observed on paper disc containing paraquat concentration as low as 20 ppm. Higher paraquat concentration caused higher inhibition effect. The most inhibited strain was *R. japonicum* strain 143.

Similar evidence is shown in Table 4, in which the diameter of inhibition zone of *Rhizobium* sp. strain G-61 and G-182 were around 2.7 cm at 100 ppm of paraquat. This stands to reason as the newly reclaimed soil has not been polluted with paraquat. *Rhizobium* sp. strain R-40 and T-37 were isolated from root nodules of Ringgit and Taichung varieties of soybean, respectively (Anonymous, 1996). These strains were not inhibited by paraquat up to 400 ppm.

The same phenomena were also shown in Table 5, i.e. inhibition of paraquat on *Rhizobium* sp. strains which were isolated from root nodules of *P. falcataria*. Some of them were inhibited by paraquat, but a strain of PF-25 was not inhibited by paraquat at least up to 400 ppm. Katayama & Kuwatsuka (1992) reported that some soil microorganisms were resistant to paraquat up to 1000 ppm. The mechanism of resistance might be due to the ability of these strains to synthesize enzymes which are responsible to paraquat detoxification (Alexander 1999, Carr *et al.*, 1985). Peroxidase enzymes could detoxify paraquat because these enzymes attack hydrogen peroxide and super-oxide substances which destroy cell membranes and disturb photosynthesis (Carr *et al.*, 1985).

Table 3. Inhibition of Paraquat on *Rhizobium japonicum*.

Rhizobium japonicum strain	Diameter (cm) inhibition zone in some levels of paraquat concentrations (ppm)				
	0	20	40	100	400
R. japonicum 78	0	1,142	1,300	1,667	[1]
R. japonicum 96	0	1,208	1,567	2,142	[1]
R. japonicum 143	0	2,492	2,883	3,408	[1]
R. japonicum 187	0	1,117	1,600	1,883	[1]
R. japonicum 194	0	1,233	1,400	1,858	[1]
R. japonicum 202	0	1,250	1,417	2,558	[1]

Note : [1] Not examined

Table 4. Inhibition of Paraquat on *Rhizobium* sp. Obtained from IIS-LIPI

Rhizobium sp. strain	Diameter (cm) inhibition zone in some levels of paraquat concentrations (ppm)				
	0	20	40	100	400
G - 60	0	1,175	1.375	1,958	[1]
G - 61	0	1,100	1,400	2,692	[1]
G - 69	0	1,142	1,550	2,617	[1]
G - 182	0	1,158	1,692	2,725	[1]
G - 183 [2]	0	1,158	1,417	2,108	[1]
G - 184	0	1,133	1,408	2,267	[1]
G - 185	0	1,117	1,433	2,233	[1]
G - 186	0	1,033	1,366	2,233	[1]
G - 199	0	1,142	1,575	2,408	[1]
G - 200	0	1,142	1,342	1,792	[1]
GSL - 48 [2]	0	1,208	1,567	2,300	[1]
GSL - 52	0	1,192	1,283	1,677	[1]
R - 40	0	0	0	0	0
R - 53	0	1,150	1,325	1,950	[1]
T - 34	0	1,175	1,417	1,867	[1]
T - 37	0	0	0	0	0

Notes : [1] Not examined; [2] *Slow grower*

Table 5. Inhibition of Paraquat on *Rhizobium* sp. Obtained from IIS-Biotechnology

Rhizobium japonicum strain	Diameter (cm) inhibition zone in some levels of paraquat concentrations (ppm)				
	0	20	40	100	400
CC – 1.1	0	1,435	1,792	2,545	[1]
KB – 1.3	0	1,192	1,425	1.908	[1]
KB – 3.3	0	1,150	1,433	1,992	[1]
PF – 2	0	1,525	1,683	1,925	[1]
PF – 3	0	1,217	1,533	1,958	[1]
PF – 16	0	1,283	1,533	2,117	[1]
PF – 20 [2]	0	1,292	1,533	1,933	[1]
PF – 25	0	0	0	0	0

Notes : [1] Not examined; [2] *Slow grower*

There is possibility that paraquat resistant strains also have the ability to use paraquat as their nitrogen and/or carbon sources, so called paraquat degrader. Martani *et al.*, (in press) isolated some bacteria which could degrade paraquat in synthetic medium. These bacteria

were isolated from Kalimantan acid sulphate soil and sapric peat soil. They were identified as *Arthrobacter* strain SM-1 and *Acinetobacter* strain S-2, respectively. The bacteria of *Achromobacter* sp., *Aerobacter aerogenes, Agrobacterium tumefaciens, Clostridium pasteureanum, Pseudomonas fluorescens* (Carr *et al.*, 1985) and the yeast of *Lipomyces starkeyi* (Carr *et al.*, 1985; Hata *et al.*, 1986) were reported as paraquat degrading microorganisms. Several researchers also showed that some species of *Rhizobium* could degrade pesticides. For example, Woodrock (1978) reported that *Rhizobium phaseoli* was able to breakdown metabolically dichlone (a chlorinated quinone group pesticide). In fact, *R. japonicum* and *R. meliloti* metabolized parathion to amino-parathion or o,o-diethyl thiophosphoric acid (Matsumura & Benzet., (1978). Therefore, there is a possibility that some of the paraquat resistant *Rhizobium sp.* strains are also degrader.

Figure 1 showed the round inhibition zone on paper discs. The zone indicates the absence of bacterial growth around paper disc containing paraquat. *Rhizobium sp.* strain CC-1.1 was inhibited by paraquat, higher concentration (from 20 – 100 ppm) caused longer diameter of inhibition zone. The diameter of zone was measured after 48 hours incubation period.

When the incubation period was prolonged until a week, some strains were able to form additional small colonies around the paper discs, especially if paraquat concentration is low (20 or 40 ppm). These phenomena showed the possibility of a growth recovery process of the bacteria treated with paraquat. Recovery process could be caused by the mutation process of *Rhizobium* which has high resistance to paraquat. Paraquat inhibition to *Rhizobium* sp. happened during a short period only. Carr *et al.* (1986) reported that *lag*-phase of *Lipomyces starkeyi* in medium containing paraquat was longer than those in non-paraquat medium. However, after that, acceleration of growth was detected due to the paraquat degradation by this soil microorganism (Carr *et al.*, 1986). Based on this phenomenon, there was a possibility that paraquat inhibited some strains of *Rhizobium* only for some days and after that, these strains would recover and started degrading paraquat. Therefore, after a week some colonies grew around the paper discs containing paraquat, especially at low concentration. However, the detail mechanism is still unknown.

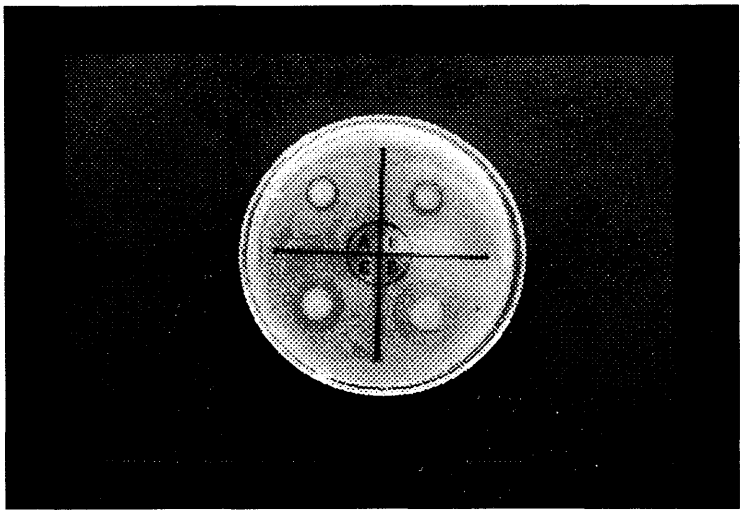

Figure 1. Inhibition of paraquat on *Rhizobium* sp. CC-1.1 on YMA added with Congo red and incubated for 48 hours. Paraquat concentrations were 0 ppm (A); 20 ppm (B); 40 ppm (C) or 100 ppm (D).

The results of this study showed that in laboratory scale experiment, paraquat inhibited most of the *Rhizobium* sp. Only six strains of *Rhizobium* (17,14%) resistant to paraquat at least up to 400 ppm. These strains could be applied as rhizobial inoculum for legume in pesticide polluted soil. Twenty nine strains out of the used strains (82.86%) were inhibited by paraquat as low as 20 ppm, higher concentration resulting in the higher inhibition effect. Some of them showed recovery in their growth, especially at 20 and 40 ppm of paraquat. Enzymatic mechanisms may responsible to the neutralization of paraquat toxicity (Carr, 1986).

Due to the uncontrolled-environmental conditions, the results obtained in laboratory scale were not always found in natural environments. Paraquat in liquid culture medium directly contacted and affected microbial cells. In soil, paraquat would be adsorbed by clay minerals and/or organic substances, so that it would be inactive to target and non-target organisms (Greenland & Hayes, 1981). Therefore, the influence of paraquat to microorganisms would be higher in culture media than that in soils.

Even still in laboratory scale, results of this study could be used as indication of the negative impact of paraquat, especially on *Rhizobium* sp. This species is only one example of thousands soil microorganisms play an important role in the nutrient cycle. Disturbances in their population may decrease environmental quality and soil health.

IV. CONCLUSIONS

Several conclusions could be obtained from this research, i.e.:

1. Application of pesticides must be controlled due to its negative impacts on non-target microorganisms.
2. In a laboratory scale experiment, paraquat inhibited the growth of *Rhizobium* sp. Higher concentration of paraquat caused higher inhibition effect.
3. Growth of the most *Rhizobium* strains (82,86 %) were inhibited by paraquat as low as 20 ppm. The other strains were resistant to paraquat, at least up to 400 ppm.
4. Resistance of *Rhizobium* sp. to paraquat was not the same for each strain, i.e. *R. japonicum* strain 143 was the most sensitive, while *Rhizobium* sp. strain PF-25, R-40, T-37, KQH, KQF and QF were resistant to paraquat.

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