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Potential *Pseudomonas* Isolated from Soybean Rhizosphere as Biocontrol against Soilborne Phytopathogenic Fungi

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Plants are liable to be attacked by soilborne fungal pathogens which are responsible to reduce plant growth and losses in yield. In Indonesia, indigenous soybeans' rhizobacteria such as antifungal producing *Pseudomonas* sp. have not many been reported yet. Therefore, the potential of the *Pseudomonas* sp. as biocontrol agent should be deeply explored. The aim of this study was to screen the indigenous soybeans' rhizobacteria *Pseudomonas* sp. that possessing biocontrol characters against soilborne mainly i.e. *Sclerotium rolfsii, Fusarium oxysporum,* and *Rhizoctonia solani, in vitro* and *in planta*. Eleven isolates identified *Pseudomonas* sp. CRB numbered by CRB-3, CRB-16, CRB-17, CRB-31, CRB-44, CRB-75, CRB-80, CRB-86, CRB-102, CRB-109, and CRB-112 were affirmed to be candidates of biocontrol agents toward the soilborne fungal pathogens. *Pseudomonas* sp. CRB inhibited growth of the pathogenic fungi approximately 11.1-60.0% *in vitro*. Among of them, 7 isolates were also produced siderophore, 2 isolates produced chitinase, and 4 isolates produced hydrogen cyanide. Seed coating with the *Pseudomonas* sp. CRB accomplished disease suppression *in planta* about 14.3-100% in sterile soil condition and 5.2-52.6% in non sterile soil condition. Consistency in high performance more than 30% of disease suppression in non sterile soil condition suggested that 5 isolates i.e. CRB-16, CRB-44, CRB-86, CRB-102, and CRB-109 isolates have great promising to be developed as biocontrol agents of soilborne pathogenic fungi.

Key words: rhizobacteria, *Pseudomonas* sp., biocontrol traits, disease suppression, soybean plant

INTRODUCTION

Modern day crop protection relies heavily on the use of chemical pesticides (Cook *et al.* 1996). Increased concern for health and environment hazards associated with the use of these agrochemicals has resulted in the need for greater sustainability in agriculture. In disease-suppressive soil and compost, disease suppression is achieved without the use of chemical (Alvarez 1995). Disease suppression in these fields is often correlated with the presence of increased numbers of antagonistic bacteria in the soil. The mechanisms by which these rhizobacteria mediate disease suppression have been investigated extensively (Thomashow & Weller 1995; Cook & Baker 1996; Haas & Keel 2003). These beneficial rhizobacteria can be developed as biological pesticides to reduce the use of chemical pesticides in agriculture; as complementary role in an Integrated Pest Management system which includes both modern environmentally safe chemical and biological strategies.

The biocontrol abilities of such strain depend essentially on the production of diffusible or volatile antifungal metabolites, aggressive root colonization, and induction of systemic resistance in the plant. Well-characterized antifungal metabolites with biocontrol properties in *Pseudomonas* spp. include phenazines, 2,4 diacetylphloroglucinol, pyoluteorin, pyrrolnitrin (Raaijmaker *et al.* 1997), and hydrogen cyanide (Haas & Keel 2003). Hydrogen cyanide is a potent inhibitor of cytochrome *c* oxidase and several other metalloenzymes, hence pathogen may suffer from deleterious effects (Blumer & Haas 2000). Low-molecular-weight compounds being called siderophore is produced to competitively acquire ferric ion. Those bacteria will deprive pathogenic fungi of this essential element since the fungal siderophores have lower affinity (O'Sullivan & O'Gara 1992). The rhizobacteria are also capable of producing lytic enzymes such as chitinases and β-1,3 glucanases (Saad 2006). These enzymes are involved in the breakdown of fungal cell walls by degrading cell wall constituents such as glucans and chitins, resulting in the destruction of pathogen structures or propagules.

Soilborne pathogenic fungi reside in the soil for brief or extended periods and survive on plant residues or as resting organisms until root exudates reach them and allow them to grow (Dickinson 2003). They either remain inside

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the plants until the host death or move outside the plants to infect other part of the root or other root. They obtain nutrients from living host tissue, reduce plant vigour and yield through the diversion of nutrients for their own growth and development (Parbery 1996). Soilborne fungal pathogens that mostly involved in agricultural practice were *Fusarium*, *Phytophthora*, *Pythium*, dan *Rhizoctonia* (Sullivan 2004). In Indonesia, *Sclerotium rolfsii*, *Fusarium* spp., and *Rhizoctonia solani* are still mentioned as primarily causing soilborne fungal disease in soybean. Plants infected by soilborne pathogenic fungi appear the disease symptoms such as seedling dumping off, cotyledon and hypocotyls damages, root rot, stem base rot, vascular wilt, or stunted. In this study, we described the *Pseudomonas* sp. isolated from the rhizosphere of soybean plant that is potential as biocontrol of soilborne phytopathogenic fungi.

MATERIALS AND METHODS

Microorganisms and Culture Condition. Total of 81 isolates from rhizosphere of soybean plant was used in this study. Identification of those isolates based on morphological and physiological characters. Gram negative, rods, motile, aerobic, catalase positive, and oxidize positive were the characters that lead to genus *Pseudomonas* identification (Holt *et al.* 1994). Microgen™ system GNA and GNB (Microgen Bioproducts Ltd, UK) that employs 24 standardized biochemical substrates in micro well was also used to complete the test of the *Pseudomonas* sp. isolates. Furthermore, 16S rRNA gene sequences of the several prospective isolates confirmed as *Pseudomonas* (Susilowati *et al.* 2010). During times of active use *Pseudomonas* sp. CRB were routinely cultivated on agar plate of King's B Medium B (KBM) (King *et al.* 1954), at room temperature. The KBM agar composition (g/l) was bacto peptone 20 (Difco, France); K_2HPO_4 1.5, $MgSO_4$ 1.5; Glycerol 15 ml/l; and agar 15. Soilborne pathogenic fungi *S. rolfsii*, *Fusarium oxysporum* (obtained from Department of Plant Protection, Bogor Agricultural University, Indonesia) and *R. solani* (obtained from Soil Research Institute, Bogor, Indonesia) were cultured on Potato Dextrose Agar (PDA) (Merck, Germany) at room temperature for 3-5 days.

Fungal Inhibition Assay *In Vitro*. Inhibition of the soilborne pathogenic fungi i.e. *S. rolfsii*, *F. oxysporum*, or *R. solani* by the *Pseudomonas* sp. CRB was performed on PDA. Bacteria were grown overnight in KBM, and each culture was streaked 3 cm from the center of the plate. One hour later, a 5 mm diameter of circular plug from an actively growing fungal culture of each pathogenic fungus placed on the surface of fresh PDA medium at the center according to Anjaiah *et al*. (1998). The inhibition of the fungal growth was determined after 3 days incubation in room temperature for *S. rolfsii*, 5 days for *F. oxysporum*, and 2 days for *R. solani*, when the mycelium growth has reached almost the edge of 9 cm Petri dish of the opposite site without the bacterial streak. Inhibition was expressed as percentage of inhibition growth of the fungi caused by the isolates (Keel *et al.* 1996). The percentage of inhibition radial growth (PIRG) was measured with the formula adopted from Dikin *et al*. (2006) as follows: PIRG (%) = [1-(length of fungal growth near to bacterial isolate/length of fungal growth other side at the same plate as control)] x 100%.

Siderophore Production. Chrome azurol S (CAS) agar plate assay was used to test for production of siderophore as described by Alexander and Zuberer (1991). Each of the *Pseudomonas* sp. CRB was streaked on CAS agar plates medium and incubated at 28 °C for 5 days. The *Pseudomonas* sp. CRB exhibiting an orange halo was considered positive for siderophore production.

Chitinase Production. Chitinase production was determined as described by Cattelan *et al*. (1999) in define medium composed of (g/l): colloidal chitin prepared from crab shell 0.8; NH_4NO_3 0.78; K_2HPO_4 0.20; $MgSO_4 \cdot 7H_2O$ 0.20; $CaCl_2$ 0.06; NaCl 0.10; $Na_2MoO_4 \cdot 2H_2O$ 0.002; $ZnSO_4 \cdot 7H_2O$ 0.00024; $CuSO_4 \cdot 5H_2O$ 0.00004; $CoSO_4 \cdot 7H_2O$ 0.010; $MnSO_4 \cdot 4H_2O$ 0.003; $Na_2FeEDTA$ 0.028; H_3BO_3 0.005 (Merck) and agar 15. Magnesium sulfate and $CaCl_2$ were autoclaved separately and added to the medium after autoclaving. Biotin (5 µg/l) and ρ-aminobenzoic acid (10 µg/l) were filter-sterilized and were added to the medium after autoclaving. Each of the isolates was spotted on the chitin medium and incubated in 28 °C for 2-3 days. Clear zone around the colony indicated chitin-solubilizing by chitinase producing bacteria.

Cyanogenesis. Hydrogen cyanide production by *Pseudomonas* sp. was detected by alkali picric method as previously described by Alvarez *et al*. (1995), Angulló (2001), and Ramette *et al*. (2003). Each of the bacterial culture was transferred into individual agar slant containing KBM supplemented with glycine (4.4 g/l). A piece of filter paper impregnated with 0.5% picric acid and 2% sodium carbonate solution was placed in the test tube. The agar slants were incubated at room temperature for 3-5 days. A change in color from yellow to orange-brown on the filter paper indicated the production of cyanide.

Analysis of Disease Suppression *In Planta*. The disease suppression of *Pseudomonas* sp. CRB toward pathogenic fungi can be related to its resistance to disease development following an artificial infestation with the homogenous mycelium of soilborne pathogenic fungi i.e. *S. rolfsii*, *F. oxysporum*, or *R. solani*.

Inoculums Preparation. Inoculum of pathogenic fungi was prepared by growing 1 cm circular plug of actively growing fungal culture in Potato Dextrose Broth (Himedia, India) supplemented with antibiotic rifampicin 50 µg/ml for 1 week on a reciprocal shaker at low speed. The mycelium was harvested with a sieve, rinsed twice with sterile distillated water, weighted and homogenized in a blender (Büttner *et al.* 2004). The amount of inoculums (cfu/ml) was determined by serial dilution and plating method. The soil was inoculated with homogenous mycelium of the pathogenic fungi that reach 10^3 cfu/g of soil.

Seed Treatment. Bacterial cultures used for seed treatment were grown as a lawn on KBM in standard Petri-dish. After 24 h at room temperature, plates were flooded with NaCl 0.85%. Cells were scraped into a centrifuge tube, washed twice by centrifugation to remove residual metabolites. Sedimented bacteria were mixed with 0.5% carboxyl methylcellulose (CMC) (BDH, England) and applied to soybean seeds. This procedure was adopted from Bonsall *et al.* (1997) and Huang *et al.* (2004). Typically, one plate of bacteria and 4.5 ml CMC suspension was use to treat 4 g of surface-sterilized seeds with the concentrations of the bacteria were 10^7-10^8 cells/ml. Control seed received only 0.5% of CMC. Ultisol soil, sand, and compost in the composition 2:1:1 were used throughout this work. Soil sterilization was carried out using autoclave, on two consecutive days, for 1 h each time. The 24 of soybean seeds were sown 1 cm deep in seedling tray containing the infested soil, and watered twice a day. Experiments were conducted under green house condition at 28-32 ^oC, in two replicates. Disease suppression (DS) was evaluated 1 week after seedling emergence by number of healthy plants, according to the formula as described by Wiyono (2003): $DS(\%) = ((X - C^+)/(C^- - C^+)) \times 100\%$. Where: X= number of healthy plants in the treatments; C^- = number of healthy plants in non infected control; C^+ = number of healthy plants in infected control.

RESULTS

Screening for Biocontrol Properties. *Pseudomonas* sp. CRB that are potential as biocontrol of the soilborne pathogenic fungi has been screened from the soybeans' rhizosphere. Among of the 81 isolates, 11 of them showing strongly inhibited the growth of soilborne pathogenic fungi i.e. *S. rolfsii*, *F. oxysporum*, or *R. solani in vitro* (Figure 1).

Antagonism of *Pseudomonas* sp. CRB against pathogenic fungi showed 11.1-60.0% of inhibition radial growth of the pathogenic fungi in plate agar. Several *Pseudomonas* sp. CRB also produced high affinity of iron chelator designated as siderophore, lytic enzyme such as chitinase and hydrogen cyanide that assumed support biocontrol performance. The isolates that posses one or more of these characteristics were advantages since it might influence against pathogenic fungi by several mechanisms. These importance biocontrol properties of the *Pseudomonas* sp. CRB are shown in Table 1.

Disease Suppression. Data obtained in Table 2 indicated that seed coating with certain *Pseudomonas* sp. CRB in artificial infected soil with *S. rolfsii*, *F. oxysporum*, or *R. solani* in an amount of 10^3 cfu/g of soil reduced the occurrence of the disease. Seed coating with *Pseudomonas* sp. CRB accomplished disease suppression

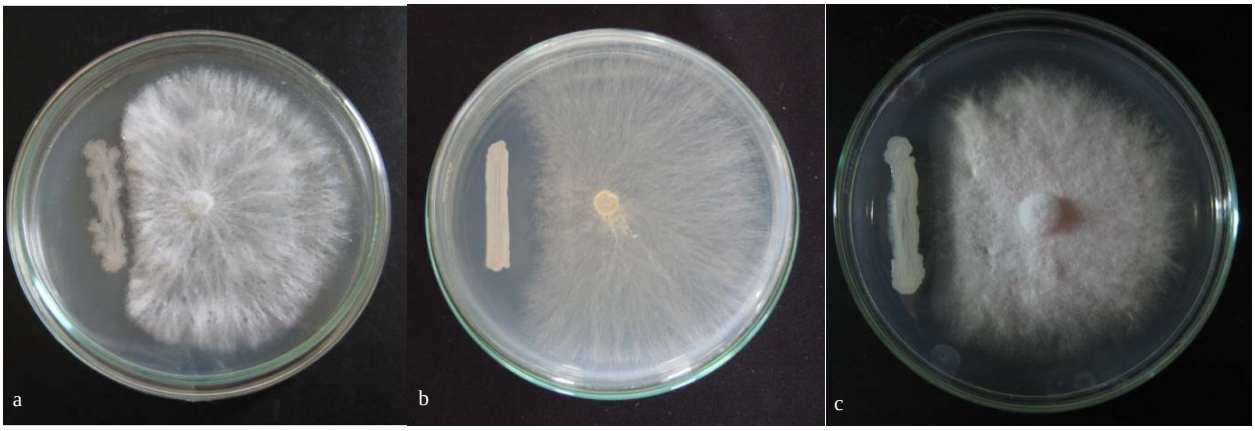

Figure 1. *Pseudomonas* sp. CRB-80 showed antagonize toward *Sclerotium rolfsii* (a), *Pseudomonas* sp. CRB-102 antagonize toward *Rhizoctonia solani* (b), *Pseudomonas* sp. CRB-86 antagonize toward *Fusarium oxysporum* (c).

Table 1. Biocontrol properties of *Pseudomonas* sp. CRB isolated from the rhizosphere of soybean plant

Name of isolate	PIRG*			Siderophore producer	Chitinase producer	Cyanogen
	S. rolfsii	*F. oxysporum*	*R. solani*			
CRB-3	-	-	56.7	+	+	+
CRB-16	-	24.6	-	+	-	-
CRB-17	-	14.3	-	+	-	-
CRB-31	-	18.7	50.0	+	-	+
CRB-44	-	39.2	-	+	-	-
CRB-75	-	11.1	37.7	-	-	+
CRB-80	20.0	-	52.3	+	+	-
CRB-86	-	30.3	36.9	-	-	-
CRB-102	25.0	-	60.0	-	-	-
CRB-109	-	-	36.9	-	-	-
CRB-112	-	-	48.1	+	-	+

*PIRG: percentage of inhibition radial growth of the fungi by the isolate. +: yes, as producer of the substance, -: no, not as producer of the substance.

about 14.3-100% in sterile soil condition. *Pseudomonas* sp. CRB-80 showed highest disease suppression about 60% toward *S. rolfsii*. The producing siderophore isolates, *Pseudomonas* sp. CRB-16, CRB-17, and CRB-44 showed better disease suppression toward *F. oxysporum* compared to non producing isolate *Pseudomonas* sp. CRB-86. *Pseudomonas* sp. CRB-102 and CRB-109 demonstrated the highest disease suppression toward *R. solani*. However, seed coating with *Pseudomonas* sp. CRB-3 in artificial infected soil with *R. solani* showed no reduction of disease occurrence as compared to the control even though it showed strong antagonism in the Petri dish. The soybean seedling damage and become stunted by terrible infection of this fungus due to the high level of fungus inoculums in the soil. *Pseudomonas* sp. CRB-31 and CRB-75 demonstrated slight disease suppression toward *R. solani* about 28.5 and 14.3% respectively.

In non sterile soil, disease suppression by *Pseudomonas* sp. CRB was about 5.2-52.6%. It was likely to become less than in sterile soil. *Pseudomonas* sp. CRB-17 showed inconsistently performance. Disease suppression by the *Pseudomonas* sp. CRB-17 toward *F. oxysporum* was highest (100%) in sterile soil but decreased into the lowest (15.7%) in non sterile soil. Other isolates also showed in reducing disease suppression. They were *Pseudomonas* sp. CRB-80 toward *S. rolfsii*; CRB-16 and CRB-44 toward *F. oxysporum*; CRB-31, CRB-102, CRB-109, and CRB-112 toward *R. solani*. Even though, there were reducing in disease suppression, some of the *Pseudomonas* sp. i.e. CRB-16, CRB-44, CRB-86, CRB-102, and CRB-109 were still able to maintain high performance more than 30% in disease suppression.

DISCUSSION

Naturally, occurring *Pseudomonas* sp. CRB could be isolated from soybean plant rhizosphere. Among of them performed antagonism toward the soilborne pathogenic fungi. A well-known and widely used assay for detection of antagonistic bacteria toward pathogenic fungi is dual culture method. This assay allow us to determine for first time whether the isolate capable to inhibit growth of the pathogenic fungi. In most cases, the evaluation of *in vitro* antifungal activity is the prerequisite for *in planta* evaluation of its antifungal activity. These antagonisms of the isolates indicated potential use for biological control of plant fungal disease. The widely recognized mechanisms of biological control mediated by rhizobacteria are including antibiotics, iron-chelating siderophore, lytic enzyme, and biocide volatile. Tree lines evidence substantiate the importance of antibiotic, for example 2,4 *diacetylphloroglucinol* production in biological control resumed by Gardener *et al.* (2001). First, mutation in the biosynthetic pathway resulted in reduced biocontrol activity. Second, the population size of 2,4 *diacetylphloroglucinol* producers in the rhizosphere correlated with disease suppressiveness of the soil and in situ antibiotic production. Third, diverse 2,4 *diacetylphloroglucinol* producing *Pseudomonas* spp. have been isolated from the rhizosphere of various crop plants. Thus inhibition zone that indicated the production of antibiotic in the *Pseudomonas* sp. CRB are important character of the isolates. The population and diversity of these isolates in the rhizosphere might facilitate in the soilborne fungal diseases suppression.

According to O'Sulivan and O'Gara (1992), bacterial siderophore, which have a very high affinity for ferric ion, are secreted during growth under low-iron condition. The resulting ferric-siderophore complex is unavailable to other organisms, but producing strain can utilize the complex of ferric-siderophore via specific receptor in its outer cell membrane. In this way, siderophore producing bacteria may restrict the growth of deleterious bacteria and fungi at the plant root. These iron starvation condition may also prevent the germination of fungal spore. Reviewed by Compant *et al*. (2005), a variety of

Table 2. Disease suppression by *Pseudomonas* sp. CRB in soybean seedlings that are grown in the *Sclerotium rolfsii*, *Fusarium oxysporum*, or *Rhizoctonia solani* infested soils (10^3 cfu/g soil)

Treatment	Sterile soil			Non sterile soil		
	No. plant with the symptom	No. healthy plant	Disease suppression (%)	No. plant with the symptom	No. healthy plant	Disease suppression (%)
S. rolfsii + CRB-80	4	20	60.0	12	12	25.0
S. rolfsii + CRB-102	8	16	20.0	13	11	31.2
S. rolfsii	10	14		16	8	
Control (without pathogen)	-	24		-	24	
F. oxysporum + CRB-16	2	22	66.6	9	15	52.6
F. oxysporum + CRB-17	-	24	100.0	16	8	15.7
F. oxysporum + CRB-44	1	23	83.3	9	15	52.6
F. oxysporum + CRB-86	5	19	16.7	10	14	47.3
F. oxysporum	6	18		19	5	
Control (without pathogen)	-	24		-	24	
R. solani + CRB-3	7	17	-	12	12	36.8
R. solani + CRB-31	5	19	28.5	16	8	15.7
R. solani + CRB-75	6	18	14.3	14	10	26.3
R. solani + CRB-102	1	23	85.7	17	7	10.5
R. solani + CRB-109	1	23	85.7	13	11	31.5
R. solani + CRB-112	3	21	57.1	18	6	5.2
R. solani	7	17		19	5	
Control (without pathogen)	-	24		-	24	

microorganisms also exhibit hyper parasitic activity, attacking pathogens by excreting cell wall hydrolases such as chitinase. Another mechanism involved in disease suppression is production of hydrogen cyanide (Flaishman *et al.* 1996; Laville *et al.* 1998). Direct inhibition of the fungi by HCN is thought to be the main mechanism of action (Blumer & Hass 2000). HCN inhibits the therminal cytochrome *c* oxidase in the respiratory chain and binds to metalloenzyme. Therefore, the *Pseudomonas* sp. CRB that have these biocontrol traits was able to give one or more mechanisms to reduce the growth of the soilborne pathogenic fungi. *Pseudomonas* sp. CRB that possessed more than one characters are interestingly since them provided by many mechanisms involved in biocontrol of pathogenic fungi. They demonstrated the ability to inhibit plant pathogenic fungi *in vitro* by indicating inhibited zone.

Almost of the eleven *Pseudomonas* sp. CRB showed disease suppression except the CRB-3 in the sterile condition. Inconsistent performance of disease suppression by antagonists bacterial in sterile and non sterile soil was not surprisingly since several researches showed the same circumstance (Scheuerell *et al.* 2005; Shishido *et al*. 2005). Inconsistent performance by bacterial antagonist has been attributed to the presence of the previous microbial in non sterile soil. They might interfere in interactions between the bacterial antagonists with non target organisms, rhizosphere competence by the bacterial antagonists and population level of the target pathogens. Competition with the previous existence microbial was more likely to have less disease suppression by the *Pseudomonas* sp. CRB. Disease control of soilborne plant pathogens in the sterile and non sterile soil condition has given variable results. However, some isolates of the *Pseudomonas* sp. i.e. CRB-16, CRB-44, CRB-86, CRB-102, and CRB-109 have maintained good performance in disease suppression more than 30% in non sterile soil condition. Based on this criterion, these isolates could be considerate as promising candidate for further application in biocontrol soilborne pathogenic fungi. Another research has also reported regarding variable results in disease suppression. Boer *et al*. (2003) found that suppressed fusarium wild by *Pseudomonas putida* WCS and induced systemic resistance by *P. putida* strain RE8 showed disease suppression 30% for the single strain treatments, but significantly enhanced to approximately 50% when WCS-358 and RE8 were mixed through soil together.

Disease suppression *in planta* showing by the isolates designated having role in diseases suppression. In this assay, they reduced number of plant with the symptom of soilborne fungal diseases and gave high percentage of diseases suppression in particular isolates. This disease suppression by the isolates suggesting that mechanisms of disease suppression against the soilborne fungal disease were related to antagonism through antifungal metabolites production, competition with the pathogens, and induced systemic resistance by bacterial siderophore. In previous research reported that bacterial siderophore involved in disease suppression by induce systemic resistance mechanisms of the plant (Bakker *et al*. 2007; Vleesschauwer *et al*. 2008).

In line with this finding, Raaijmaker *et al*. (1997) resumed that, numerous strains of antibiotic-producing fluorescent *Pseudomonas* spp. were readily isolated from soils that are naturally suppressive to disease such take-all of wheat, black root rot of tobacco or fusarium wilt of tomato, indicating that they may play an important role in the natural biological control that occur in these soils. *Pseudomonas* species indigenous to the soil and plant such as *Pseudomonas* sp. CRB can be assumed have a major role in diseases suppression when they send back to the rhizosphere of soybean plant. Therefore, from this study, the isolates offered promising to be developed as biocontrol agents of phytopathogenic fungi to protect the soybean plant from the diseases caused by the fungi.

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