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Pectobacterium carotovorum Inhibition by Preservative agents in Sprouting Radish Seeds

Atchara Chatkaew, Mukhamad Su'udi, Sooyeon Lim, Byung-Ho Hwang and Jongkee Kim*
Department of Integrative Plant Science, Chung-Ang University, Anseong 456-756, KOREA

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Abstract

In the present study, we report the effect of preservative agents against Pectobacterium carotovorum ssp. carotovorum (Pcc), the causal agent of soft rot disease, on sprouting radish seeds. Compounds were mixed with nutrient agar at concentrations of 0.002 M, 0.02 M and 0.2 M. In vitro assay showed that out of ten compounds, sodium metabisulfite and sodium sulfite were able to inhibit bacterial growth at all concentrations. In addition, the reduction of bacterial population was in agreement with increasing holding time and concentration of compounds. In vivo assay of both compounds also exhibited similar tendency, in which, high concentration shows more inhibition effects on bacterial growth. The population of Pcc on radish sprout after treated with sodium metabisulfite and sodium sulfite were 0.00 and 6.68 log_{10} CFU/ml, compared to the control (7.06 log_{10} CFU/ml). However, the sodium metabisulfite has stronger negative effects on seed germination compare to sodium sulfite at 0.2 M. High concentration of both compounds also interfere the seedling elongation and fresh weight. The results indicate that appropriate amount of both compounds might be used for controlling the Pcc growth.

Keywords: *Pcc, soft rot, Raphanus sativus L*

Introduction

Radish (*Raphanus sativus* L.) is a root vegetable crop consumed by a majority of Asian people. Among all parts of mature radish plant, root was mostly used for daily food or side-dishes. In addition, seedling of radish (sprout) contain many kinds of secondary metabolites compounds such as glucosinolate[1], vitamin C, anti-oxidant[2] and any other small molecules that contribute to human health[3]. One of the small molecules found in radish is isothiocyanate, which has been reported to have beneficial effects against cancer cells in human[4].

However, the radish production is restricted by several numbers of environmental conditions such as biotic and abiotic stresses. One example of biotic disturbance is caused by fungal or bacteria. *Pectobacterium carotovorum* ssp. *carotovorum* (*Pcc*) is one example of economically important bacteria infected the radish and other vegetable. This pathogen can reduce the vegetable yield, and therefore, has been included in the top ten bacterial pathogens infected plants[5].

During the growing, harvesting, packaging and distribution processes, the radish might be contaminated with bacteria[6]. Several sanitation methods have been applied to prevent and reduce the contamination problem. These methods including the physical treatment such as high pressure[7,8], heat treatment[7], ozone exposure[9], electron beam and gamma irradiation[10,11]. Several chemical compounds were also used for sanitation purpose. These chemical usually have salt or acid properties such as chlorine dioxide[12], sodium hypochlorite[13], fumaric acid[12] and acidic electrolyzed water[14]. One of commercial chemical used for sanitation is chlorine bleach. However, this compound might harmful for environments and generate very strong odor[15].

Recently, several preservative agents have been reported to have an inhibition effects against bacterial growth. In the previous report, the preservative agents can induce the stress responses in microorganism[16,17]. The preservative agents such as alum and lime have been reported to decrease the infection of soft rot in cabbages[18]. In addition, salt compounds were effective to reduce *Pcc* in potato tuber[19].

This study was conducted to observe the effect of preservative agents on *Pcc* growth. The effect of preservative agents against *Pcc* was also determined on radish seeds and sprouts. The side effects of preservative agents treatments on seed germination and sprouting is discussed.

Material and Methods

Plant Materials: The radish cultivar (cv.) Chungwoonplus was used in this study. Five hundred grams of radish seeds were sterilized through soaking in 1% of sodium hyperchlorite for 1 min, 70% ethanol for 20 sec, and then washed with deionized water as described[20]. The seeds were air dried inside the biosafety hood at 25ºC until uniformly dry.

Bacterial Culture and Seed Inoculation: The *Pectobacterium carotovorum* ssp. *carotovorum* (*Pcc*) was streaked on Nutrient agar[21,22] and was incubated at 28°C for 24 hours. A single colony was picked up and added into 10 ml of Nutrient broth (NB), and incubated overnight on shaking incubator at 28°C,

150 rpm. One ml of overnight bacterial seed culture was transferred to 100 ml of fresh NB medium and was incubated at 28°C for 24 hours. The cell suspension (10^9 CFU/ml) was mixed with the radish seeds at ratio of 5:3 (v/v) for 5 min. The cell suspension was removed from seeds with sterilized cheesecloth. The seeds were dried inside the biosafety hood at 25ºC for 30 min. The seeds (2 grams) were wrapped with sterilized cheesecloth and were prepared for next analysis according to the method described by Neetoo and Chen[8].

Preservative Agent for Screening Analysis: The preservative agents (acetic acid, citric acid, oxalic acid, magnesium chloride, magnesium nitrate, potassium carbonate, potassium nitrate, sodium carbonate, sodium sulfite and sodium metabisulfite) were added to NA plate (pH 6.5) at three different concentrations, 0.002 M, 0.02M and 0.2 M. NA plate without any chemical compound was used as the control. The bacterial stock solution was grown in NB at 28°C for 24 hours, 1 ml of cell culture was added to 9 ml of 0.1% sterile peptone water to prepare 10^{-5} dilution. The aliquot (100 µl) of cell suspension was spread plated on NA using a sterile cell spreader. The plates were incubated at 28ºC for 3 days.

Optimizing Time for *Pcc* Inactivation: The compounds and cell suspension were mixed in 9:1 (v/v) ratio. At 0 time incubation, 1 ml of mixture was directly diluted with sterile 0.1% peptone water for 10^{-1} - 10^{-7} serial dilution. For 5, 10, 15 and 20 min incubation time, the mixture were incubated at indicated times and was carried as mentioned above. The 100 µl of each diluted bacterial suspension was spread plated on NA using a sterile cell spreader and incubated at 28ºC for 3 days.

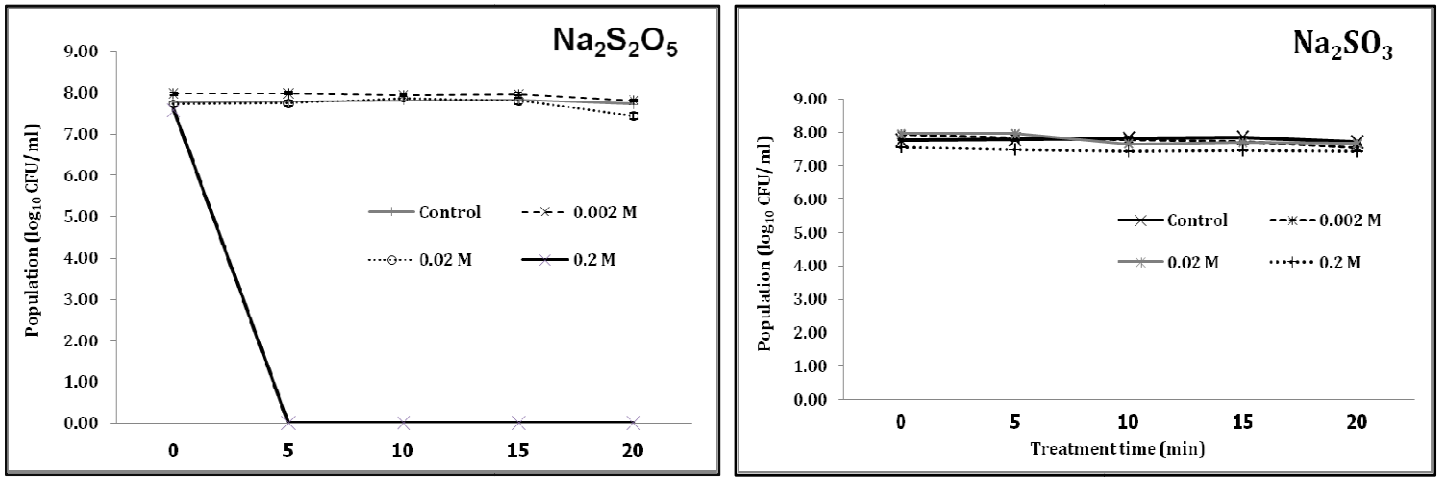
Microbial Population on Seeds: The sodium metabisulfite concentration and incubation were 0.2 M for 5 min, 0.02 M for 20 min and 0.002 M for 20 min, while for sodium sulfite were 0.2 M for 5 min, 0.02 M for 10 min and 0.002 M for 20 min. Sterilized water was used as control. The infected seeds were randomly selected and spread on wet paper towels on the plastic rack and then placed in the chamber. Two grams of seeds were mixed with 18 ml of 0.1% peptone water for 2 min with agitation (260 rpm). 1 ml of mixture was directly diluted with sterile 0.1% peptone water and processed as described above. The plates were incubated in chamber at 28ºC for 3 days.

Seed Germination, Length and Fresh Weight: From two grams infected seeds, 100 seeds were randomly selected and spread on the layers of wet paper towels on the plastic rack. The moisture condition on paper towels were maintained by spraying distilled water every 6 hours. The percentages of germinated were determined after 0, 1, 2 and 3 from the onset of germination. Each compound at each concentration was tested with three replications per experiment. The percentages of germination were calculated as the total number of germinating seeds to the total number of seeds. The lengths of sprouts were determined every day using digi-caliper (Cienceware®, U.S.A). The seeds (2 grams) were germinated on the layers of wet paper towels with maintained moisture condition on the bucket. The fresh weight was determined every day (0, 1, 2 and 3 days). The fresh weight was calculated as described previously[23].

Results and Discussion

Growth Inhibition of *Pcc* in Media Containing Preservative Agents: The growth inhibition of *Pectobacterium carotovorum* ssp. *carotovorum* (*Pcc*) by several preservatives agents are summarized in table-1. At the concentration of 0.002 M, only sodium sulfite and sodium metabisulfite showed complete inhibition effects on bacterial growth. At moderate concentration (0.02 M), the acetic acid, potassium carbonate and sodium carbonate also inhibited *Pcc* growth. The effects of several preservative agents were significantly difference ($P \leq 0.05$) at those both concentrations. However, at high concentration (0.2 M), all preservative agents were able to cease *Pcc* growth (0 $\log_{10}$ CFU/ml). In previous reports, low concentrations of organic acids were effectively inhibits the microbial growth. The application of acid condition in the food has been reported to have preventive effects to microbial growth[24]. At higher concentration, organic acids have been used for sweep over the microorganisms[25]. Similarly, Gurtler et al[26] reported that high concentration of organic acids can inhibit the activity of bacteria. In our experiment, at concentration of 0.02 M, only one acidic molecule (acetic acid) and four salt molecules (potassium carbonate, sodium carbonate, sodium sulfite and sodium metabisulfite) showed full bacterial growth inhibition.

Optimizing Time for *Pcc* Inactivation: Since the sodium sulfite and sodium metabisulfite shown significant inhibition for *Pcc* growth, we conducted further experiment to determine the effect of incubation time. As shown in figure-1, there were no different of *Pcc* growth in media without preservative agents (control) and with sodium sulfite and sodium metabisulfite. However, at concentration of 0.2 M, the *Pcc* growth in media containing sodium metabisulfite was completely inhibited (0 $\log_{10}$ CFU/ml) at 5 minutes incubation time and further (figure-1A). In contrast, in the media containing the same concentration of sodium sulfite, the *Pcc* growth was slightly inhibited at all incubation times (figure-1B). This result is in agreement with previous reports for the positive correlation between preservative compound concentration and incubation (holding) times[8]. Trinetta et al[27] also observed that high concentration of preservative agents was able to inactivate food borne pathogens more effectively. Our results further confirmed the previous report of Mills et al[19], in which the application of sodium metabisulfite can reduced the population of *Erwinia* spp. Furthermore, Roberts and McWeeny[28] reported that sulfur dioxide was effective against gram-negative rods bacteria such as *E. coli* and *Pseudomonas*, but less effective to the gram-positive rods bacteria such as *Lactobacillus*. In addition, Basaran-Akgul et al[29] stated that sulfur dioxide was effective to reduce the bacterial population as low as 5-log by reaction with several essential macromolecules (protein carbonyl groups, FAD^+, RNA and DNA).



(A) (B)

Figure-1
Growth of *Pcc* in nutrient agar treated with sodium metabisulfite (A) and sodium sulfite (B) at three different concentrations (0.2 M, 0.02 M and 0.002 M) and different inoculation times (0, 5, 10, 15 and 20 min)

***Pcc* Inhibition by Preservative Agents on Radish Seeds:** In the next, we tested the effects of preservative agent in the radish seeds after pre-treated with *Pcc*. The results for sodium metabisulfite at concentration of 0.2, 0.02 and 0.002 M were 0.00, 5.88 and 6.85 log_{10} CFU/ml, respectively, at initial day (0 day). Complete *Pcc* growth inhibition caused by this chemical at concentration of 0.2 M was remains persistent up to 2 days. In contrast, for sodium sulfite, the colony forming units were 6.68, 7.19 and 7.04 log_{10} CFU/ml at concentration of 0.2, 0.02 and 0.002 M, respectively (table-2). In both compounds, except at concentration of 0.2 for sodium metabisulfite, bacterial population was increase at all concentrations after treatment at 0 day until the final day (3 days). The *Pcc* population in the final day in the control was 9.14 log_{10} CFU/ml, while at 0.2 M sodium metabisulfite and sodium sulfite were 7.91 and 9.26 log_{10} CFU/ml, respectively. In the present study, sodium metabisulfite and sodium sulfite were effective to reduce the *Pcc* growth *in vivo*. There are several possibilities for the mechanism of the sulfite ion to inhibit the bacterial cell growth. The sulfite ion could enter the cell membrane and disrupts the normal metabolic activity of bacterial cells[30]. In addition, the decreasing pH caused by the presence of sulfite ion might inhibit bacterial growth and respiration[31]. In the previous study using fungal pathogen, the sodium metabisulfite could increase the permeability of cell membrane through destruction of membrane lipids[32]. Similarly, Mecteau et al[33] reported that the sodium metabisulfite has the ability to inhibit mycelial growth and spore germination completely and reduced the development of dry rot disease in potato tuber.

Effect of Preservative Agents Treatment on Radish Seed Germination, Seedling Elongation and Fresh Weight: Table-3 summarized the germination percentage of radish seeds after treated with sodium metabisulfite and sodium sulfite. In the first 24 hours (first day), at concentration of 0.2 M sodium metabisulfite and sodium sulfite, the germination percentage were 0.33% and 24.33%, respectively, compared to 68.00% in the control. At the second day, the germination was 8.00% and 54.67% for seeds treated with sodium metabisulfite and sodium sulfite, respectively. Meanwhile, the mock treated seeds germination was 81.00%. At the final day, the germination percentage for seeds treated with 0.2 M sodium metabisulfite and sodium sulfite were increased up to 23.00% and 68.00%, while in the control was 85.00%. On the other hand, the germination percentages at the concentration of 0.02 M and 0.002 M were approximately 90.00%. These indicate that at high concentrations of both compounds somewhat interfere the germination process. In contrast, low concentrations of these compounds were not restrains the seed germination progression, as comparable with mock control (table-3). Previous study by Li et al[34] showed that low pH effectively inhibit bacterial growth. However, this condition also gave some negative effects to seed germination. In addition, salt solution at high concentration might delay the seed germination due to osmotic pressure effect[35]. Other researchers have also observed the effect of salt and osmotic stresses on seed germination, but not seedling development[36]. This osmotic stresses might decrease the water uptake during imbibitions because of high salt concentration which probably evoked excessive uptake of ions[37].

The data for seedling elongation of radish seed after treated with sodium metabisulfite and sodium sulfite was summarized in table-4. At first day, all treatment at 0.2 M of sodium metabisulfite and sodium sulfite showed shorter seedling than other treatment. The lengths of seedling generated from seed after treated with sodium metabisulfite and sodium sulfite were 4.68 and 6.95 mm. At second day, the seedling length raised from sodium metabisulfite treatment was 4.88 mm, extremely different with other treatment. At third day, there was no significant different ($P \leq 0.05$) for all compounds except 0.2 M of

sodium metabisulfite. The length of seedling was 8.12 mm, about 12 times shorter than control. Our study demonstrates that, all compounds have no effects for seedling elongation at all concentration, except 0.2 M of sodium metabisulfite (table-4). The fresh weight was significantly influenced ($P \leq 0.05$) by preservative agents treatment, as shown in table-5. The fresh weight was increased gradually following the germination times. The lowest fresh weight was observed in 0.2 M sodium metabisulfite treatment at 3 days after germination. The fresh weight was 3.16 g, about 1.5 times lower than control. This study shows that the concentration of compounds govern both germination and growth rate. In accordance with previous study by Khan et al[38], high salinity inhibits the seed germination at concentrations beyond the tolerance limits of the species. Kaya et al[35] also mentioned that salt solution has effect on seedling growth rather than the germination from osmotic effect. In the case of different fresh weight between sodium metabisulfite and sodium sulfite treatments, it should be due to the different in the seed capability for absorbing water and minerals. The similar mechanism has been reported by Mao-Jun et al[39].

Table-1
Growth of *Pcc* in nutrient agar added with ten different preservative agents at three different concentrations (0.2 M, 0.02 M and 0.002 M) and incubated at 28°C for 3 days

Compound	*Pcc* (log_{10} CFU/ml)		
	0.002 M	**0.02 M**	**0.2 M**
Control	8.14^{b}	8.14^{c}	8.14^{a}
Acetic acid	7.78^{d}	0.00^{f}	0.00^{b}
Citric acid	8.48^{a}	8.48^{a}	0.00^{b}
Magnesium chloride	8.15^{b}	7.81^{d}	0.00^{b}
Magnesium nitrate	8.48^{a}	8.18^{b}	0.00^{b}
Potassium carbonate	7.99^{c}	0.00^{f}	0.00^{b}
Potassium nitrate	8.15^{b}	7.72^{e}	0.00^{b}
Sodium carbonate	8.41^{a}	0.00^{f}	0.00^{b}
Sodium sulfite	0.00^{e}	0.00^{f}	0.00^{b}
Sodium metabisulfite	0.00^{e}	0.00^{f}	0.00^{b}
Oxalic acid	8.48^{a}	8.48^{a}	0.00^{b}
C.V. (%)	0.57	0.36	0.41
F-test	**	**	**

Data represents the mean values of colony forming unit of *Pcc* on log survivors (CFU/ml). Within a column, different letters indicate significant ($P \leq 0.05$).

Table-2
Growth of inoculated *Pcc* on radish seeds treated with sodium metabisulfite (0.2 M for 5 min, 0.02 M for 20 min and 0.002 M for 20 min) and sodium sulfite (0.2 M for 5 min, 0.02 M for 10 min and 0.002 M for 20 min)

Compound	Time (min)	Bacterial population on seed (log10 CFU/ml)			
		0 Day	**1 Day**	**2 Days**	**3 Days**
Control	0	7.06^{b}	8.46^{a}	9.25^{ab}	9.14^{a}
0.2 M $Na_2S_2O_5$	5	0.00^{f}	0.00^{e}	0.00^{e}	7.91^{b}
0.02 M $Na_2S_2O_5$	20	5.88^{e}	7.95^{d}	9.06^{c}	9.17^{a}
0.002 M $Na_2S_2O_5$	20	6.85^{c}	8.17^{c}	9.23^{ab}	9.26^{a}
0.2 M Na_2SO_3	5	6.68^{d}	8.24^{b}	9.30^{a}	9.26^{a}
0.02 M Na_2SO_3	10	7.19^{a}	8.46^{a}	9.20^{b}	9.25^{a}
0.002 M Na_2SO_3	20	7.04^{b}	8.28^{b}	8.88^{d}	9.24^{a}
C.V. (%)		1.21	0.43	0.57	0.77
F-test		**	**	**	**

Data represents the mean values of colony forming unit of *Pcc* on log survivors (CFU/ml). Within a column, different letters indicate significant ($P \leq 0.05$).

Table-3
Percentage of seed germination treated with sodium metabisulfite (0.2 M for 5 min, 0.02 M for 20 min and 0.002 M for 20 min) and sodium sulfite (0.2 M for 5 min, 0.02 M for 10 min and 0.002 M for 20 min)

Compound	Time (min)	Percentage of radish seed germination (%)			
		0 Day	1 Day	2 Days	3 Days
Control	0	0.00^{a}	68.00^{b}	81.00^{a}	85.00^{a}
0.2 M $Na_2S_2O_5$	5	0.00^{b}	0.33^{d}	8.00^{c}	23.00^{c}
0.02 M $Na_2S_2O_5$	20	0.00^{c}	70.67^{ab}	86.67^{a}	92.00^{a}
0.002 M $Na_2S_2O_5$	20	0.00^{d}	75.00^{ab}	86.00^{a}	91.67^{a}
0.2 M Na_2SO_3	5	0.00^{e}	24.33^{c}	54.67^{b}	68.00^{b}
0.02 M Na_2SO_3	10	0.00^{f}	77.00^{a}	85.33^{a}	91.00^{a}
0.002 M Na_2SO_3	20	0.00^{g}	78.67^{a}	85.33^{a}	90.67^{a}
C.V. (%)		0.00	8.09	7.41	5.19
F-test		ns	**	**	**

Data represents the mean value of percentage of seed germination. Within a column, different letters indicate significant ($P \leq 0.05$).

Table-4
Seeds elongation treated with sodium metabisulfite (0.2 M for 5 min, 0.02 M for 20 min and 0.002 M for 20 min) and sodium sulfite (0.2 M for 5 min, 0.02 M for 10 min and 0.002 M for 20 min)

Compound	Time (min)	Seeds elongation (mm)			
		0 Day	1 Day	2 Days	3 Days
Control	0	4.13^{b}	8.16^{b}	35.90^{a}	97.61^{a}
0.2 M $Na_2S_2O_5$	5	4.68^{a}	4.68^{c}	4.88^{c}	8.12^{b}
0.02 M $Na_2S_2O_5$	20	4.36^{ab}	9.55^{a}	24.34^{b}	83.20^{a}
0.002 M $Na_2S_2O_5$	20	4.28^{ab}	10.06^{a}	29.58^{ab}	95.23^{a}
0.2 M Na_2SO_3	5	4.28^{ab}	6.95^{b}	25.91^{b}	78.66^{a}
0.02 M Na_2SO_3	10	4.41^{ab}	9.81^{a}	20.46^{b}	74.95^{a}
0.002 M Na_2SO_3	20	4.27^{ab}	9.71^{a}	23.77^{b}	85.46^{a}
C.V. (%)		7.25	12.35	27.22	29.93
F-test		ns	**	**	**

Data represents the mean value of length of sprouting seeds. Within a column, different letters indicate significant ($P \leq 0.05$)

Table-5
Fresh weight of sprouting seeds treated with sodium metabisulfite (0.2 M for 5 min, 0.02 M for 20 min and 0.002 M for 20 min) and sodium sulfite (0.2 M for 5 min, 0.02 M for 10 min and 0.002 M for 20 min)

Compound	Time (min)	Fresh weight (g)			
		0 Day	1 Day	2 Days	3 Days
Control	0	2.03^{a}	2.99^{b}	3.90^{d}	4.95^{a}
0.2 M $Na_2S_2O_5$	5	2.04^{a}	2.91^{c}	2.98^{f}	3.16^{e}
0.02 M $Na_2S_2O_5$	20	2.03^{a}	2.92^{c}	3.97^{c}	4.82^{c}
0.002 M $Na_2S_2O_5$	20	2.03^{a}	3.03^{a}	4.06^{b}	4.90^{ab}
0.2 M Na_2SO_3	5	2.03^{a}	2.87^{d}	3.42^{e}	4.02^{d}
0.02 M Na_2SO_3	10	2.03^{a}	2.97^{b}	3.98^{c}	4.92^{a}
0.002 M Na_2SO_3	20	2.03^{a}	2.99^{b}	4.28^{a}	4.85^{bc}
C.V. (%)		0.41	0.62	0.53	0.88
F-test		ns	**	**	**

Data represents the mean value of fresh weight. Within a column, different letters indicate significant ($P \leq 0.05$)

Conclusion

The present study shows that among ten preservative agents, sodium metabisulfite and sodium sulfite were effective to inhibit *Pcc*. Both compounds effectively reduce the cell population number at high concentration and longer incubation time. Although both compounds inhibit symptom development of radish seedling infected with *Pcc*, the sodium metabisulfites shows stronger inhibition effects. On the other hand, high concentration of sodium metabisulfite has negative effects on seed germination, seedling elongation and fresh weight. Since these compounds are not harmful for human consumption, the results of this study might be useful for application in the food processing, especially inhibition of microbial growth.

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