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EFFECT OF ANTIBIOTIC FORMULA ON QUALITY AND HUANGLONGBING IN YONGXING 'BINGTANG' SWEET ORANGE ORCHARDS

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ABSTRACT

Huanglongbing (HLB), caused by the obligate phloem-limited bacterium *Candidatus* Liberibacter spp., is one of the most devastating diseases threatening global citrus production. In this study, 'Bingtang' sweet orange (*Citrus sinensis* (L.) Osbeck) was used as the experimental material to evaluate the efficacy of different antibiotic formulations for HLB management and their impacts on fruit quality from May to August 2023. The results showed that the tested antibiotic treatments significantly reduced *C*Las titers and exerted transient inhibitory effects on HLB pathogens with no detectable antibiotic residues. Additionally, antibiotic-treated 'Bingtang' sweet orange fruit exhibited a pronounced increase in soluble solid content compared with the control group. This study provides a scientific basis for the rational application of antibiotics in HLB management.

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INTRODUCTION

Citrus (*Rutaceae, Citrus*) ranks among the most widely cultivated and abundantly produced fruit crops worldwide. Valued for its desirable sweet–sour taste and rich nutritional profile, citrus fruit enjoys extensive consumer popularity (Ramana et al. 1981; Liu et al. 2012; Peris et al. 2019). Citrus fruits accumulate abundant carbohydrates, vitamins, and minerals, as well as diverse bioactive compounds including alkaloids, flavonoids, and carotenoids. These functional components contribute substantially to human health promotion and disease prevention (Lv et al. 2015; Sohi et al. 2018; Ma et al. 2024). Citrus prefer warm and humid climates and exhibit a lengthy phenological cycle, which makes them susceptible to a wide range of pests and diseases at all growth stages. In the majority of citrus-producing regions, HLB represents the most severe biotic constraint, leading to substantial yield losses and compromised fruit quality. (Zhou 2020; Ammar et al. 2020; Bassanezi et al. 2020). Currently, no effective cure is available for HLB. Its high transmissibility has resulted in severe economic damage to the global citrus industry (Bové 2006; Gottwald 2010).

HLB is caused by the uncultur-able, Gram-negative bacterium *Candidatus* Liberibacter spp., which is taxonomically classified under the phylum Proteobacteria, class α-proteobacteria, order Rhizobiales, family Rhizobiaceae, and genus Liberibacter (Jagoueix et al. 1994; Bové 2006; Ghosh et al. 2018). On the basis of geographical origin and 16S rDNA sequence variations, three distinct *Candidatus* Liberibacter species are responsible for HLB, including *Ca*. L. asiaticus (*C*Las), *Ca*.

L. africanus (*C*Laf), and *Ca*. L. americanus (*C*Lam) (Bové 2006; Chen et al. 2011).*C*Laf is thermosensitive and endemic to Africa, whereas *C*Las exhibits stronger heat tolerance and originates from Asia, with a broad distribution spanning Asia, Africa, the Arabian Peninsula, and the Americas. *C*Lam is another heat-tolerant strain that is exclusively restricted to Brazil. Among the three HLB-associated pathogens, *C*Las is the most prevalent and destructive strain. It has invaded nearly all major citrus-growing regions worldwide, excluding the Mediterranean Basin and Australia (Jagoueix et al. 1994; Teixeira et al. 2005). HLB pathogens are mainly transmitted via two pathways: grafting propagation in field production and infestation by the citrus psyllid vector (Gottwald et al. 2007).

In China, citrus cultivation has a history of more than 4,000 years. Currently, HLB has spread to over 300 counties across ten major citrus-producing provinces, severely hindering the sustainable development of the citrus industry (Zhou 2018). Recently, a growing body of research has explored alternative mitigation strategies, including phytohormone-mediated host resistance induction (Singh et al. 2022; Nehela & Killiny, 2023), nutritional optimization (Vashisth & Grosser, 2018; Vashisth & Livingston, 2019; Atta et al. 2022), thermotherapy (Lopes et al. 2009; Hoffman et al. 2013), soil amendment application (Xu et al. 2013), and exogenous regulation of plant growth (Zheng et al. 2013; Shahzad et al. 2024). Among these, antibiotic has attracted particular attention.Previous studies have demonstrated that trunk-injected antibiotics can significantly alleviate HLB symptoms and reduce bacterial titers (Zhang et al. 2014; Hu et al. 2017). Collectively, these findings indicate that antibiotics hold considerable potential for HLB management. However, while antibiotics can effectively suppress the pathogen, complete eradication has not been achieved under field conditions. Consequently, removal of symptomatic trees and replanting with pathogen-free seedlings remains the ultimate measure for controlling disease spread (Gottwald et al. 2010; Hall et al. 2011; Bassanezi et al. 2020). Yet this strategy is not only time-consuming but also imposes heavy economic losses on growers. Further exploration of optimized antibiotic applications is warranted to mitigate disease impact while preserving fruit yield and quality. To this end, the present study was designed to evaluate the effects of several antibiotic formulations, applied via trunk injection and foliar spraying, on HLB-infected citrus trees, along with comprehensive assessments of fruit quality and antibiotic residues, with the aim of providing a basis for establishing a scientifically robust and practically feasible integrated HLB management strategy.

MATERIALS AND METHODS

Research Area and Condition

The field trial was carried out from May to August 2023 in commercial orchards of 'Bingtang Pao No.1' sweet orange in Yongxing County, Chenzhou City, Hunan Province, China. All experimental trees were cultivated inside an insect-proof screenhouse. Yongxing County lies at coordinates 26.127151° N, 113.116528° E, with an annual mean precipitation of roughly 1500 mm and an average annual temperature of 19 °C.

Application of Treatments

In this experiment, a total of 35 HLB-infected sweet orange trees were subjected to antibiotic treatments. Six antibiotic combination treatments (A, B, C, D, E, F) and one distilled-water control group (K) were established, with 5 replicate trees per treatment (Table 1). All treatments were initiated in May, with a one-month application interval. For trunk injection, each tree was treated using a drip bag fitted with two needles: one inserted deeply into the xylem and the other shallowly into the phloem, delivering a total volume of 200 mL solution per tree. Foliar spraying, supplemented with fungicides and insecticides, was performed approximately 15 days after each trunk injection depending on weather conditions. In total, four rounds of foliar applications were conducted, with a cumulative spray volume of 15 L per treatment group.

Leaves Sample Collection, DNA Extraction and qPCR

Leaf samples were harvested from 35 HLB-infected sweet orange trees, with uniform branches permanently tagged for consistent sampling. The presence of *Candidatus* Liberibacter asiaticus was verified via quantitative polymerase chain reaction (qPCR). Briefly, 0.1 g of fresh leaf midrib tissue was collected for genomic DNA extraction using the CTAB method described by Murray & Thompson (1980). DNA quality and concentration were determined using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies Inc., USA). The qPCR assay was performed using the forward primer HLBas (TCGAGCGCGTATGCAATACG), reverse primer HLBr (GCGTTATCCCGTAGAAAAAGGTAG), and fluorescent probe HLBp (AGACGGGTGAGTAACGCG). The

20 μL reaction system contained 10 μL of TSE301 2× T5 Fast qPCR Mix (Beijing Tsingke Biotech Co., Ltd., China), 0.8 μL of forward primer, 0.8 μL of reverse primer, 0.2 μL of probe, 1 μL of DNA template, 0.4 μL of RoxII normalization reagent, and nuclease-free water to reach the final volume. The qPCR thermal profile consisted of an initial denaturation at 95 °C for 5 min (1 cycle), followed by 40 cycles of 95 °C for 10 s and 65 °C for 50 s.

Table1. Different treatments and formulation.

Treatments	Method	Application Time	Application of Formulation and Working Concentrations
A	Trunk Injection	May, June, July, August	Ampicillin 100-fold dilution + Amoxicillin 100-fold dilution + 0.1% Triacontanol 1000-fold dilution + Medium-Element Water-Soluble Fertilizer 1000-fold dilution
B	Trunk Injection	May, June, July, August	Ampicillin 500-fold dilution + Amoxicillin 500-fold dilution + 0.1% Triacontanol 1000-fold dilution + Medium-Element Water-Soluble Fertilizer 1000-fold dilution
C	Trunk Injection	May, June, July, August	Ampicillin 1000-fold dilution + Amoxicillin 1000-fold dilution + 0.1% Triacontanol 1000-fold dilution + Medium-Element Water-Soluble Fertilizer 1000-fold dilution
D	Trunk Injection	May, June, July, August	Ampicillin 100-fold dilution + Amoxicillin 100-fold dilution + 0.1% Triacontanol 1000-fold dilution + Medium-Element Water-Soluble Fertilizer 1000-fold dilution
	Foliar Spray	May	20% Thiodiazole-copper 300-fold dilution + 20% Dinotefuran 2000-fold dilution
		June	30% Cupric nonyl phenolsulfonate 500-fold dilution + 25% Thiamethoxam 2000-fold dilution
		July	47% Kasugamycin copper oxychloride wettable powder 600-fold dilution + 22% Sulfoxaflor 2000-fold dilution
		August	3% Zhongshengmycin 800-fold dilution + 70% Imidacloprid 3000-fold dilution
E	Trunk Injection	May, June, July, August	Ampicillin 500-fold dilution + Amoxicillin 500-fold dilution + 0.1% Triacontanol 1000-fold dilution + Medium-Element Water-Soluble Fertilizer 1000-fold dilution
	Foliar Spray	May	20%Thiodiazole-copper 300-fold dilution + 20%Dinotefuran 2000-fold dilution
		June	30% Cupric nonyl phenolsulfonate 500-fold dilution + 25% Thiamethoxam 2000-fold dilution
		July	47% Kasugamycin copper oxychloride wettable powder 600-fold dilution + 22% Sulfoxaflor 2000-fold dilution
		August	3%Zhongshengmycin 800-fold dilution + 70% Imidacloprid 3000-fold dilution
F	Trunk Injection	May, June, July, August	Ampicillin 1000-fold dilution + Amoxicillin 1000-fold dilution + 0.1% Triacontanol 1000-fold dilution + Medium-Element Water-Soluble Fertilizer 1000-fold dilution
	Foliar Spray	May	20% Thiodiazole-copper 300-fold dilution + 20% Dinotefuran 2000-fold dilution
		June	30% Cupric nonyl phenolsulfonate 500-fold dilution + 25% Thiamethoxam 2000-fold dilution
		July	47% Kasugamycin copper oxychloride wettable powder 600-fold dilution + 22% Sulfoxaflor 2000-fold dilution
		August	3% Zhongshengmycin 800-fold dilution + 70% Imidacloprid 3000-fold dilution
K	Control	May, June, July, August	Distilled water

Analysis of Antibiotic Residues in Fruits

Fruit sampling was conducted at 7, 25, 60, and 74 days after each treatment to dynamically monitor antibiotic residue levels, with ten fruit samples collected per time point. Antibiotic residues were quantified according to the method reported by Ren et al. (2018). Briefly, homogenized fruit

samples were mixed with 10 mL of 25% aqueous acetonitrile solution. The mixture was ultrasonicated for 30 min, fully vortexed, and centrifuged at 8000 r/min for 10 min. The resulting supernatant was filtered through a 0.22 μm microporous membrane and subsequently determined using an Agilent 1290-6470 liquid chromatography–mass spectrometry (LC-MS) system.

Physical and Biochemical Analysis of Fruit Samples

Six fruits were randomly sampled from each treatment for fruit quality determination. Single-fruit weight was measured using an electronic balance. A Vernier caliper was used to determine the equatorial and longitudinal fruit diameters, as well as equatorial peel thickness. Peel color parameters were detected at four equatorial diagonal positions of each fruit using a MINOLTA CR-300 colorimeter (Japan) based on the CIELAB color space. The soluble solids content (SSC) was determined with a PAL-1 handheld digital refractometer (ATAGO, Japan).

Data Analysis

All experimental data were organized using WPS 2024 and statistically analyzed via SPSS 26.0. One-way analysis of variance (ANOVA) was performed, and Duncan's multiple range test was used to identify significant differences among treatment groups.

RESULTS

Effect of Antibiotic Treatments on Huanglongbing Infection

Leaf samples were consistently collected from fixed branches of experimental trees for HLB pathogen detection after different antibiotic treatments. qPCR detection showed that HLB pathogens were completely undetectable in all trees from treatments A and B, and in 1, 1, 3, and 3 individual trees from treatments C, D, E, and F, respectively (Table 2). In addition, 3 trees in group C, 4 trees in group D, 2 trees in group E, and 1 tree in group F exhibited CT values greater than 32, indicating substantially reduced pathogen concentrations in these trees (Table 2). In contrast, trees in the untreated control group remained persistently HLB-positive. These findings highlight the effective role of antibiotic applications in suppressing HLB infection.

Table 2. Detection of HLB in 'Bingtang' sweet orange trees before and after treatment.

Treatments	Pre-treatment		Post-treatment		Treatments	Pre-treatment		Post-treatment	
	Cycle Threshold	HLB Presence	Cycle Threshold	HLB Presence		Cycle Threshold	HLB Presence	Cycle Threshold	HLB Presence
A1	27	+	—	-	B1	35	+	—	-
A2	21	+	—	-	B2	32	+	—	-
A3	19	+	—	-	B3	29	+	—	-
A4	20	+	—	-	B4	32	+	—	-
A5	27	+	—	-	B5	24	+	—	-
C1	23	+	36	+	D1	24	+	31	+
C2	31	+	35	+	D2	22	+	36	+
C3	21	+	—	-	D3	20	+	36	+
C4	19	+	34	+	D4	20	+	34	+
C5	28	+	21	+	D5	23	+	37	-
E1	22	+	—	-	F1	25	+	21	+
E2	20	+	—	-	F2	22	+	—	-
E3	19	+	—	-	F3	23	+	39	-
E4	23	+	35	+	F4	21	+	—	-
E5	21	+	34	+	F5	30	+	19	+
K1	22	+	19	+					
K2	21	+	20	+					
K3	26	+	20	+					
K4	26	+	21	+					
K5	20	+	22	+					

"—" denotes no Ct value."+" denotes detection of HLB infection. "-" denotes no detection of HLB infection.

Antibiotic Residue Analysis

Antibiotic residue dynamics in citrus fruits were comprehensively analyzed at 7, 25, 60, and 74 days after different antibiotic treatments (Table 3). At 7 days post-application, no antibiotic residues were detected in treatment C, corresponding to the 1000-fold diluted antibiotic concentration, indicating rapid dissipation of the applied antibiotics. By 25 days, ampicillin residues were undetectable in treatments A, C, E, and F, and all treatment groups displayed a consistent downward trend in residual antibiotic levels. At 60 days post-application, neither ampicillin nor amoxicillin residues were detected in any treatment groups. Follow-up detection at 74 days further confirmed that no antibiotic residues remained in all fruit samples. These results demonstrate that optimized antibiotic treatments can effectively control HLB while avoiding persistent antibiotic residue accumulation in citrus fruits.

Effect of Antibiotic Treatment on Fruit Parameters

Compared with the control group, antibiotic treatments caused no significant differences in fruit L* values, suggesting that peel lightness was not substantially altered (Table 4; Figure 1).

Table 3. Residue levels of ampicillin and amoxicillin post-treatment.

Treatments	7 days post-treatment		25 days post-treatment		60 days post-treatment		74 days post-treatment	
	Ampicillin (μg/kg)	Amoxicillin (μg/kg)	Ampicillin (μg/kg)	Amoxicillin (μg/kg)	Ampicillin (μg/kg)	Amoxicillin (μg/kg)	Ampicillin (μg/kg)	Amoxicillin (μg/kg)
A	6.40	30.11	—	14.60	—	—	—	—
B	80.61	464.66	31.25	62.35	—	—	—	—
C	—	—	—	—	—	—	—	—
D	386.19	913.00	13.01	8.71	—	—	—	—
E	71.99	1096.36	—	6.29	—	—	—	—
F	13.01	53.80	—	7.60	—	—	—	—

"—" denotes antibiotic residues were not detected (LOD＝5μg/kg).

Table 4. Fruit parameters analysis of post-treatment.

Treatments	Luminance (L*)	Red-Green Axis (a*)	Yellow-Blue Axis (b*)	Weight per fruit (g)	Equatorial diameter (mm)	Longitudinal diameter (mm)	Equatorial skin thickness (mm)	Soluble Solid Content (%)
A	58.56±4.42a	-14.56±9.37ab	52.36±5.58bc	145.11±26.20a	66.70±5.99a	60.83±3.81a	4.29±0.38a	12.17±0.68a
B	63.52±3.02a	-4.48±4.73a	63.92±5.74a	162.59±26.32a	68.64±3.47a	65.27±3.49a	3.36±0.30a	12.37±0.06a
C	60.63±3.84a	-15.08±10.92ab	56.59±6.23abc	167.75±2.98a	70.18±1.90a	64.10±4.71a	4.09±0.76a	11.97±0.67a
D	61.18±1.23a	-13.87±1.11ab	57.99±3.11abc	167.74±30.93a	69.63±3.07a	66.30±6.37a	4.49±0.52a	12.10±1.65a
E	62.29±6.16a	-5.01±6.26a	60.49±4.48ab	147.81±18.89a	66.97±4.21a	62.35±3.92a	4.43±1.44a	12.43±0.31a
F	62.74±5.64a	-8.89±8.51ab	57.46±6.24abc	144.82±16.79a	65.85±3.49a	62.94±0.92a	4.48±0.59a	13.10±0.40a
K	56.96±5.04a	-19.97±3.39b	49.44±4.84c	155.93±17.48a	68.25±3.53a	61.57±2.98a	4.03±0.26a	9.37±0.86b

a,b Different lowercase letters indicate significant differences according to Duncan's Multiple Range Test (P ≤ 0.05).

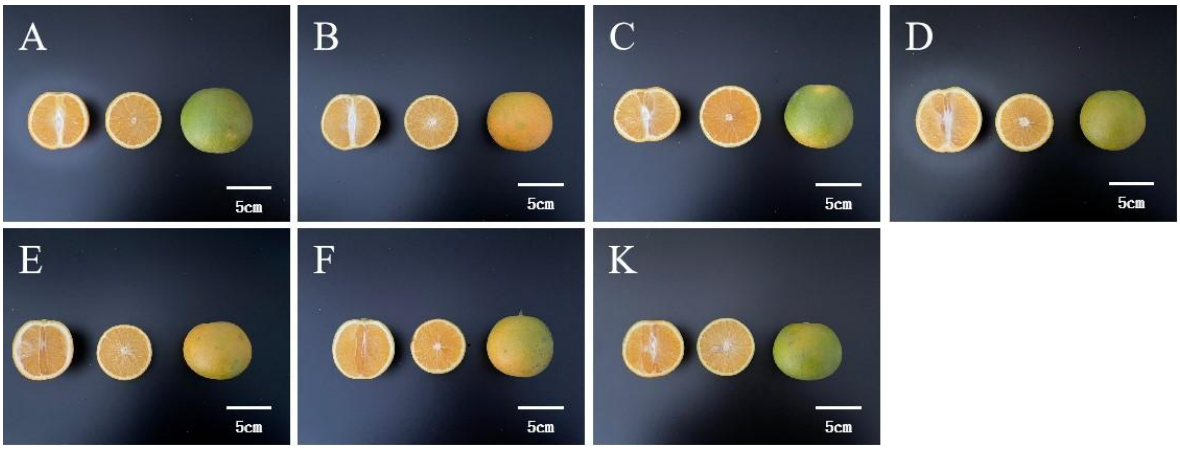


Figure 1. Appearance of 'Bingtang' sweet orange fruit.

The control group presented significantly lower a* values (red–green axis) than the antibiotic treatments, whereas no obvious differences in a* values were observed among all treated groups. For b* values (blue–yellow axis), only treatment B showed a significant increase. No significant differences in single-fruit weight, equatorial diameter, longitudinal diameter, or peel thickness were detected between the control and antibiotic treatment groups (Figure 1). Notably, the control group had the lowest soluble solids content (SSC). Although SSC did not differ significantly among antibiotic treatments, treatment F yielded the highest SSC value of 13.1% (Table 4).

DISCUSSION

'Bingtang' sweet orange, a member of the Rutaceae family and Citrus genus, is a elite local cultivar derived from natural mutation of common sweet orange (Duan et al. 2007; Cao et al. 2021). Its fruits are oblate or nearly spherical in shape. With attractive golden peel color, crisp and sweet flesh, high soluble solid content, low acidity, pleasant flavor, and few seeds, 'Bingtang' sweet orange exhibits excellent fruit quality and is widely popular among consumers. Citrus Huanglongbing (HLB), the most destructive disease threatening global citrus production (Bové 2006), has been continuously spreading alongside the expansion of citrus cultivation, severely restricting the sustainable development of the citrus industry. To date, no resistant citrus varieties or effective curative agents are available for HLB management (Zhou 2020). Accordingly, HLB is widely regarded as the "cancer disease" of citrus.

HLB is caused by a Gram-negative bacterium, and broad-spectrum antibacterial antibiotics have been verified to effectively inhibit HLB progression. As early as the 1970s, long before the clarification of HLB etiology, antibiotics were already confirmed to possess therapeutic potential against this disease (Su & Chang 1974). Subsequent field and greenhouse trials have further validated that trunk injection and root immersion can effectively suppress *C*Las proliferation to varying degrees (Zhang et al. 2011, 2012, 2013). Previous pharmacological studies have demonstrated that the combined application of ampicillin and streptomycin achieves a significantly higher reduction in *C*Las titer than single applications of ampicillin or tetracycline (Zhang et al. 2011). Moreover, trunk injection of benzbromarone, tolfenamic acid, and oxytetracycline has exhibited promising control efficacy against HLB (Ghosh et al. 2018; Li et al. 2019; Gardner et al. 2020). Huang et al. (2021) identified stable antimicrobial peptides (SAMPs) that can markedly reduce in planta *C*Las abundance via trunk injection and simultaneously activate host innate immunity to resist bacterial infection. Trunk injection boasts a core advantage in that it delivers agrochemicals directly into the phloem, thereby maximizing drug utilization efficiency and minimizing environmental contamination. Consistent with previous findings, the present study confirmed that trunk-injected antibiotic formulations (treatments A and B) exerted excellent therapeutic effects on HLB-infected 'Bingtang' sweet orange trees, achieving complete elimination of detectable *C*Las in infected tissues after treatment. Previous studies have documented that HLB infection reduces fruit soluble solids content (SSC), thereby impairing the sweetness and flavor quality of citrus fruits (Baldwin et al. 2010; Raithore et al. 2015). In agreement with these findings, our results showed that antibiotic-treated trees produced fruits with significantly higher SSC than the untreated control, further verifying the positive regulatory effect of antibiotic therapy on recovering fruit quality. Additionally, HLB infection has been reported to cause diminished fruit size and abnormal fruit morphology (Mishra et al. 2011; Baldwin et al. 2017; McCollum & Baldwin 2017). However, no significant differences in fruit weight, equatorial diameter, or longitudinal diameter were observed between the control and antibiotic treatment groups in this study. This phenomenon suggests that HLB-induced morphological defects are primarily restricted to severely symptomatic fruits (Tatineni et al. 2008), whereas mild infection exerts limited impacts on fruit morphological traits.

The agricultural application of antibiotics for plant disease management remains controversial due to widespread public concerns regarding residual risks and ecological side effects, which greatly restrict their large-scale field promotion (Zhang et al. 2014; Li et al. 2020). Nevertheless, our fruit residue analysis demonstrated that no antibiotic residues were detectable in any treatment groups at 60 days post-application. These findings indicate that the optimized antibiotic treatment strategy can effectively reduce *C*Las titers and alleviate HLB damage without causing persistent residual contamination in citrus fruits.

In conclusion, this study demonstrates that the optimized antibiotic formulations, particularly treatments A and B, can effectively mitigate HLB infection in 'Bingtang' sweet

orange with no persistent antibiotic residues in fruits. The tested antibiotic treatments significantly alleviated typical HLB symptoms and restored fruit quality, confirming their reliable efficacy for HLB control. Although such trunk-injected antibiotic strategies have not yet been widely popularized in commercial citrus production, this study provides valuable theoretical support and feasible technical references for the safe and efficient management of citrus HLB. Further systematic field trials are required to promote the large-scale application and standardized optimization of antibiotic-based HLB prevention and control techniques.

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CONFLICT OF INTEREST

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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