

Effects of Culture Method and Formulation on the Virulence of *Steinernema riobrave* (Rhabditida: Steinernematidae) to *Diaprepes abbreviatus* (Coleoptera: Curculionidae)¹

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Abstract: The Diaprepes root weevil, *Diaprepes abbreviatus*, is a pest of vegetables, ornamental plants, sugarcane, and citrus in Florida and the Caribbean. The entomopathogenic nematode, *Steinernema riobrave*, can reduce larval populations of *D. abbreviatus* substantially. Efficacy of entomopathogenic nematodes, however, may be affected by culture method and formulation. Using *D. abbreviatus* as the host, we compared the efficacy of two commercial *S. riobrave* formulations, a liquid and a water-dispersible granule (WDG), with each other and with in vivo produced *S. riobrave*. Nematodes in the commercial formulations were produced in vitro through liquid fermentation; the in vivo nematodes were cultured in *Galleria mellonella* and applied in aqueous suspension. Laboratory experiments measured nematode virulence in plastic cups containing soil and seventh-eighth instar *D. abbreviatus*. One laboratory experiment was conducted using only fresh nematodes (less than 5 days old); another experiment included WDG nematodes that were stored for 25 days at 10 °C. Two field experiments were conducted in which nematodes were applied either to potted citrus (containing *D. abbreviatus* larvae) placed beneath mature citrus trees or to soil directly beneath the tree. In the latter experiment, efficacy was determined by measuring mortality of caged *D. abbreviatus* larvae that were buried beneath the soil surface prior to application. Mortality of *D. abbreviatus* treated with nematodes ranged from 80–98% and 50–75% in laboratory and field experiments, respectively. In all experiments, we did not detect any significant effects of culture method or formulation.

Key words: biological control, citrus, culture, *Diaprepes abbreviatus*, entomopathogenic nematode, formulation, *Steinernema riobrave*.

The Diaprepes root weevil, *Diaprepes abbreviatus* (L.), is a pest of vegetables, ornamental plants, sugarcane, and citrus in Florida and the Caribbean (McCoy, 1999). Larvae of *D. abbreviatus* can cause severe damage to citrus trees by feeding on their root systems (McCoy, 1999). Due to environmental and regulatory concerns, chemical control against larvae of *D. abbreviatus* is limited (McCoy, 1999). Currently, the only recommended management practice available to control *D. abbreviatus* larvae that have established themselves in citrus groves is application of entomopathogenic nematodes (Bullock et al., 1999a).

Entomopathogenic nematodes (genera

Heterorhabditis and *Steinernema*) are biocontrol agents that kill their invertebrate hosts with the aid of a mutualistic bacterium (Kaya and Gaugler, 1993). These nematodes are amenable to mass production and have been commercialized in more than seven countries (Grewal and Georgis, 1998). Four species have been sold commercially in Florida to control *D. abbreviatus* in citrus: *H. bacteriophora* Poinar; *H. indica* Poinar, Karunakar, and David; *S. carpocapsae* (Weiser); and *S. riobrave* Cabanillas, Poinar, and Raulston.

Reported field efficacy of entomopathogenic nematodes against *D. abbreviatus* larvae has been variable (Adair, 1994; Downing et al., 1991; Duncan and McCoy, 1996; Duncan et al., 1996, 1999; Schroeder, 1987). For example, *D. abbreviatus* suppression by *H. bacteriophora* varied from 56–83% (Downing et al., 1991) to no detectable control (Adair, 1994; Duncan and McCoy, 1996).

Variation in efficacy can be caused by a variety of factors relating to the nematode, its host, and the environment (Kaya and Gaugler, 1993). For example, the virulence of several species (*H. bacteriophora*, *H. indica*,

Received for publication 14 March 2000.

¹ Florida Agriculture Experiment Station Journal Series No. R-07516. This study was supported in part by the Citrus Production Research Advisory Council, Grant No. 942-18E.

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The authors thank I. Jackson, A. Hoyte, and S. Hoobin for technical assistance, and L. Duncan and M. Abu Hatab for critical review of an earlier draft of this manuscript.

This paper was edited by P. Grewal.

and *S. riobrave*) toward *D. abbreviatus* decreases as larval age increases and as temperature decreases (i.e., reduced virulence was observed at 21 °C relative to 24 °C) (Shapiro et al., 1999). One of the most critical factors in successful application of nematodes is choosing the proper nematode species or strain for the target pest (Georgis and Gaugler, 1991). Laboratory, greenhouse, and field studies indicate that *S. riobrave* has the highest virulence among all the nematodes tested thus far against *D. abbreviatus* (Bullock et al., 1999b; Duncan and McCoy, 1996; Duncan et al., 1996; Schroeder, 1987; Schroeder, 1994; Shapiro and McCoy, 2000a, 2000b).

Entomopathogenic nematode efficacy can also be affected by culture method (Gaugler and Georgis, 1991; Grewal et al., 1999) and formulation (Baur et al., 1997). Entomopathogenic nematodes can be mass-produced by in vivo or in vitro (i.e., solid or liquid fermentation) methods (Friedman, 1990; Grewal and Georgis, 1998). In vitro liquid culture is deemed to be the most efficient method of production (Friedman, 1990). However, depending on nematode species and media composition, in vitro produced nematodes may exhibit reduced quality and efficacy (Abu Hatab et al., 1998; Abu Hatab and Gaugler, 1999; Gaugler and Georgis, 1991; Yang et al., 1997).

Regardless of culture method, once entomopathogenic nematodes are produced commercially they must be formulated for delivery and application (Georgis 1990; Georgis et al., 1995). An effective formulation provides a suitable shelf life, stability of product from transport to application, and ease of handling (Georgis et al., 1995). A breakthrough in formulation technology was cited in the introduction of a water-dispersible granule (WDG), in which the nematodes enter a partially anhydrobiotic state allowing them to survive up to 6 months at 4–25 °C (substantially longer than previous formulations) (Georgis et al., 1995). Subsequent research, however, indicated reduced efficacy of WDG-formulated *S. carpocapsae* relative to in vivo produced nematodes (Baur et al., 1997).

Two commercial formulations of *S. riobrave* are currently available for control of *D. abbreviatus* in Florida citrus—a WDG and a liquid formulation. Product labels indicate a shelf life of up to 1 month at 13 to 25 °C for the WDG, and 48 hours below 24 °C for the liquid formulation. Nematodes in both formulations are produced by in vitro liquid culture. Our objective was to determine if culture method or formulation affects the efficacy of *S. riobrave* toward *D. abbreviatus* larvae. We conducted laboratory and field comparisons of nematodes produced in vitro (WDG and liquid formulations) with in vivo produced nematodes (applied in aqueous suspensions). Comparison of the liquid formulation to the in vivo produced nematodes was used to determine culture method effects, and comparison of the liquid formulation to the WDG was used to determine formulation effects.

MATERIALS AND METHODS

Seventh and eighth instar *D. abbreviatus* were obtained from the USDA-ARS Horticultural Laboratory (Orlando, FL) and used in all experiments. *Steinernema riobrave* (Biovector® 355) were obtained from Thermo Trilog Corporation (Columbia, MD). In vivo nematode cultures were established by rearing the nematodes in larvae of the greater waxmoth, *Galleria mellonella* (L.), according to procedures described in Kaya and Stock (1997). In vivo cultures did not exceed five passages through the host, and the in vivo nematodes used in experimentation were stored at 10 °C for less than 5 days. Viability in all formulations was determined prior to application by probing nematodes under a stereomicroscope (three replicates) (Kaya and Stock, 1997).

Laboratory experiments: In this experiment, we compared fresh and stored nematode formulations under controlled conditions. Two trials were conducted to compare virulence of in vitro (liquid and WDG formulations) and in vivo (applied in aqueous) produced nematodes. Experimental units consisted of plastic containers (3.5-cm i.d., 8.5 cm deep) filled with Candler sand (percent-

age sand:silt:clay = 96.6%: 2.0%: 1.4%, pH = 6.3, organic matter = 0.3%). Soil moisture within the containers was standardized at approximately 8% by weight (ca. -0.03 bars). A single seventh or eighth instar *D. abbreviatus* was placed on the bottom of each container prior to filling with soil, and 500 infective juveniles (IJs) were applied to the soil surface. To increase nematode motility, WDG-formulated nematodes were aerated for 1 hour prior to determination of viability (Baur et al., 1997).

Experiments were arranged as completely randomized designs and were conducted at 24 °C. All treatments contained 4 replicates of 10 cups each. The first trial consisted of four treatments: in vivo, liquid, WDG, and a water control. In vitro produced liquid and WDG nematodes were from the same production batch and were stored at 10 °C for 1 day prior to experimentation. The second trial consisted of three treatments: in vivo, WDG, and a water control. WDG nematodes were stored for 25 days at 10 °C before use. In both trials, larval mortality was determined 13 days post-inoculation.

Field experiment 1: This experiment compared WDG and liquid formulations under field conditions. Nematodes were applied to the soil beneath the trees in a mature citrus grove near Fort Pierce, Florida, with conventional spray equipment (McCoy et al., 2000a). This 9-year-old grove consisted of mixed oranges on Swingle citrumelo rootstock with a 3.0 × 7.6-m setting in two row beds. Soil type was a Pineda sandy soil (94% sand) with a pH of 6.9 and organic matter content of 1.1%.

Three treatments—WDG, liquid, and a control (no nematodes)—were arranged in a completely randomized design with 10 replicates (plots) per treatment. The nematodes, which were from the same batch used in the laboratory experiments, were applied on the same day they were received from the manufacturer. Nematodes were applied to plots, which consisted of 6 adjacent trees in two rows (12 trees total), using an herbicide delivery system with twin 1.5-m booms (the system was cleaned thoroughly before use).

Nematodes were applied as a band on both sides of the tree at a water volume of 153 liters/ha and a speed of 2.4 km/hour. Nematodes were applied at a rate of 54 IJs/cm². The number of nematodes applied was calculated based on the quantity listed on the manufacturer's label. We took this approach because we wanted to apply nematodes in a manner similar to how citrus growers apply them. To facilitate nematode survival and penetration into the soil, microsprinkler irrigation was applied for at least 1 hour prior and 1 hour post application according to procedures described by McCoy et al. (2000).

Nematode efficacy was estimated by determining mortality of caged *D. abbreviatus* larvae (McCoy et al., 2000a). These cylindrical cages were made of 225 stainless steel mesh (7 × 3-cm diam.) with snap-on polypropylene caps on each end. In each plot, one cage with a single larva was placed randomly 12–20 cm below the soil surface under each of four center trees at approximately 1–1.3 m from the trunk. Cages were placed in the grove approximately 3 hours before nematode application and were removed 7 days later, at which time larval mortality was recorded.

Field experiment 2: This experiment compared liquid (in vitro) with in vivo nematodes in potted citrus seedlings. Pots consisted of polyvinyl chloride pipe (15.2 × 15.2-cm diam.) with an open top and 225 steel mesh bottom (McCoy et al., 2000b). Each pot contained soil common to the grove (the same field described in the first experiment) and one rootstock citrus seedling (Sun Shu Sha), which was kept in the greenhouse for 1 month prior to nematode application in order to allow the root systems to develop. Ten *D. abbreviatus* larvae were added to each pot 3 days prior to nematode inoculation.

Nematodes were applied at a rate of 54 IJs/cm². All liquid formulated nematodes, which were from a different batch than in the other experiments, were applied on the same day they were received. Nematode rates were determined via serial dilution,

and the dose intended for each pot (in a 7-ml water suspension) was added to a 15-ml conical polystyrene centrifuge tube (Corning, Oneonta, NY). Nematodes were immediately transported under refrigeration to the grove for application. Nematodes were then applied by pouring the contents of one centrifuge tube onto the soil in each nematode treatment pot (controls received an equal volume of water).

The experiment consisted of three treatments in which the pots remained in the grove following nematode application: in vivo (in aqueous) nematodes, liquid (in vitro) nematodes, and a control (water). For comparison (as a kind of positive check), an additional set of control pots, and pots treated with in vivo nematodes, were returned to the greenhouse (25–28 °C) immediately following application. Each treatment consisted of 20 replicates (i.e., 100 pots total). Following nematode inoculation, one pot from each of the three treatments that remained in the field was placed approximately 1.3 m from the tree. The pots were placed at a depth of approximately 12 cm, and Insect Trap Coating (Tanglefoot Corp., Grand Rapids, MI) was applied to the exposed edge of the pot to prevent predator invasion (e.g., ants) (McCoy et al., 2000a). Microsprinkler irrigation was applied as in the first field experiment. Nine days after nematode inoculation, soil was removed from all pots and the number of *D. abbreviatus* remaining was recorded.

In order to suppress endemic entomopathogenic nematode populations in the field (Duncan et al., 1999; McCoy et al., 2000a) the experimental area was treated with NemaCur® (37 kg/ha). According to the manufacturer's label, *S. riobrave* should not be applied to an area treated with NemaCur® for 2 weeks. To be prudent, we allowed a 1-month period to pass between the applications of NemaCur® and *S. riobrave*. Immediately prior to application of *S. riobrave*, the effect of NemaCur® was evaluated by extracting nematodes on modified Baermann funnels according to methods described by Duncan et al (1996); nematode density in untreated soil (from the adjacent

row) was compared to NemaCur®-treated soil (20 replicates of four soil cores each). Additionally, the potential of residual effects (1 month after NemaCur® application) on *S. riobrave* was tested using the laboratory assay described previously; 500 *S. riobrave* were applied to soil cups containing untreated or treated soil, and *D. abbreviatus* mortality was determined 5 days later (4 replicates).

Statistical analysis: Mean *D. abbreviatus* mortality was compared among treatments with analysis of variance (ANOVA). If treatment effects were detected with ANOVA, the differences among treatments were elucidated with the SNK test (SAS Institute, Cary, NC). The effects of NemaCur® (1 month after application) in treated vs. untreated soil were determined using T-tests (SAS Institute, Cary, NC).

RESULTS

Laboratory experiments: No significant differences in *D. abbreviatus* mortality were detected among nematode treatments in either laboratory trial (Fig. 1A,B). However, larval mortality in all nematode treatments was significantly greater than the control (Fig. 1A,B). In the first trial, nematode viability averaged (\pm sd) 88.5 ± 2.1 , 75.9 ± 5.2 , and 79.0 ± 5.6 for the in vivo, liquid, and WDG nematodes, respectively. In the second trial, nematode viability averaged (\pm sd) 100 ± 0 and 64.7 ± 2.9 for the in vivo and WDG nematodes, respectively.

Field experiment 1: No significant differences in *D. abbreviatus* mortality were detected between liquid and WDG-formulated nematodes (Fig. 2). Larval mortality caused by the WDG formulation was significantly greater than in the control, whereas mortality from the liquid treatment was not different from the control (Fig. 2). Nematode viability averaged (\pm sd) 78.0 ± 4.2 and 85.7 ± 2.3 for the liquid and WDG nematode formulations, respectively.

Field experiment 2: No significant differences in *D. abbreviatus* mortality were detected between the in vitro liquid and in vivo treatments that remained in the field (Fig. 3). The in vivo treatment that was kept in

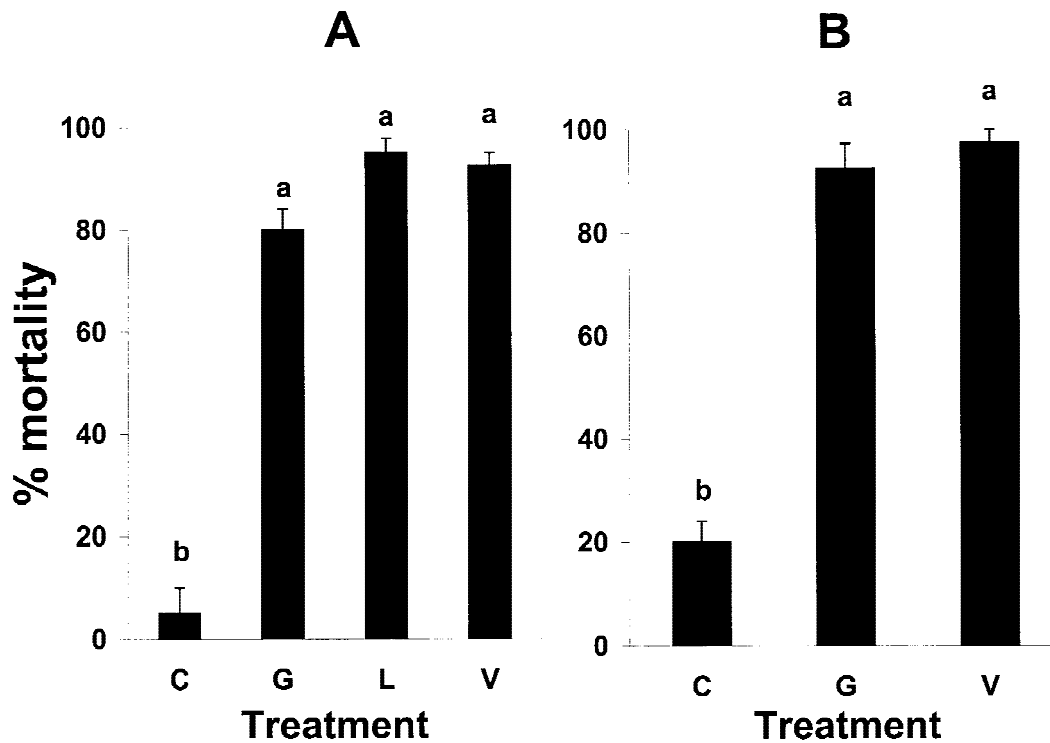


FIG. 1. Mean percentage mortality of *Diaprepes abbreviatus* larvae in the laboratory recorded 13 days after exposure to three formulations of *Steinernema riobrave*: C = control (water), G = granular formulation (in vitro produced), L = liquid formulation (in vitro), V = in vivo formulation (applied in aqueous suspension). Different letters above bars indicate statistical significance (SNK test, $P \leq 0.05$). A) Nematodes in all formulations were less than 5 days old. B) Granular-formulated nematodes were stored 25 days at 10 °C prior to experimentation; in vivo nematodes were less than 5 days old.

the greenhouse caused greater *D. abbreviatus* mortality than nematode treatments left in the grove (Fig. 3). Larval mortality was higher in all nematode treatments relative to both controls. Controls were not different from each other (Fig. 3). Nematode viability averaged (\pm sd) 100 ± 0 and 86.0 ± 5.6 for the in vivo and liquid nematode formulations, respectively. One month after Nematicur® application, endemic nematode densities were lower in Nematicur®-treated soil than in untreated soil ($P = 0.05$); mean nematode densities (per cm^2 soil) averaged (\pm se) 0.39 ± 0.09 and 0.83 ± 0.19 for the treated and untreated soils, respectively. Soil cup assays indicated that *S. riobrave* virulence to *D. abbreviatus* was not significantly affected by Nematicur® (1 month after treatment) ($P = 0.70$); *D. abbreviatus* mortality averaged (\pm se) 40 ± 8.2 and 45 ± 9.5 in the treated and untreated soils, respectively.

DISCUSSION

Baur et al. (1997) reported that *S. carpocapsae* formulated in WDG was less virulent

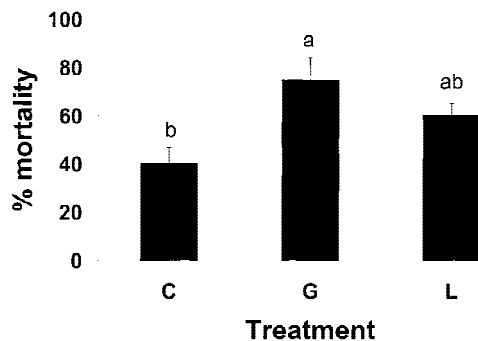


FIG. 2. Mean percentage mortality of caged *Diaprepes abbreviatus* in a mature citrus grove recorded 7 days after exposure to two formulations of *Steinernema riobrave*: C = control (water), G = granular formulation (in vitro produced), L = liquid formulation (in vitro). Different letters above bars indicate statistical significance (SNK test, $P \leq 0.05$).

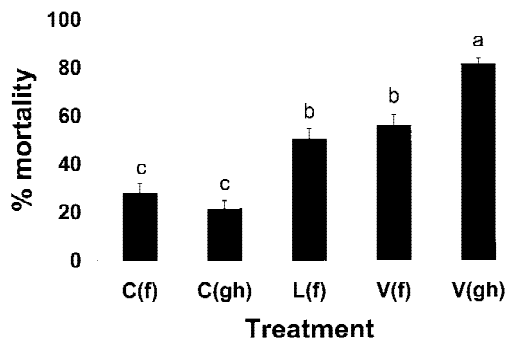


FIG. 3. Mean percentage mortality of *Diaprepes abbreviatus* in potted citrus recorded 9 days after exposure to two *Steinernema riobrave* formulations: C = control (water), L = liquid formulation (produced in vitro), V = in vivo formulation (applied in aqueous suspension), f = pots kept in a mature citrus grove, gh = pots kept in the greenhouse. Different letters above bars indicate statistical significance (SNK test, $P \leq 0.05$).

toward the black cutworm, *Agrotis ipsilon* (Hufnagel), than in vivo (*G. mellonella*) produced nematodes applied in aqueous suspension. Because no in vitro produced liquid formulation was tested, it was not possible to determine if the reduced virulence was caused by formulation or by culture method (Baur et al., 1997). We did not detect any effects of formulation or culture method on the efficacy of *S. riobrave* in laboratory and field tests reported herein. Other studies have indicated variable effects of formulation and culture method on entomopathogenic nematodes. Yang et al. (1997) reported reduced body length, weight, and motility in *S. carpocapsae* cultures produced in vitro (solid fermentation) relative to nematodes produced in vivo. Gaugler and Georgis (1991) found no effect of culture method on the efficacy of *S. carpocapsae* yet the efficacy of *H. bacteriophora*, produced in liquid fermentation, was reduced relative to in vivo produced *H. bacteriophora*. Grewal et al. (1999) also did not detect effects of culture method on *S. carpocapsae* but reported that *S. scapterisci* produced in liquid culture had higher virulence (toward *G. mellonella*) than *S. scapterisci* produced in house crickets (*Acheta domesticus*). Thus, it seems effects of culture method and formulation can depend on nematode species. Species differences also may have contributed to the di-

vergence in observations in our study compared with that of Baur et al. (1997). The effects of culture method also can depend on media composition (Abu Hatab et al., 1998; Abu Hatab and Gaugler, 1999; Han et al., 1992).

In the first field experiment, the lack of statistical significance when comparing the liquid (in vitro) formulation to the control was likely due to high endemic nematode populations (Duncan et al., 1999; McCoy et al., 2000a). In the same experiment, the *D. abbreviatus* mortality from the WDG was significantly greater than the control. This could have been due to over-packing, i.e., excess nematodes are added to each unit of WDG to ensure that at least the labeled amount of nematodes are available during the shelf life of the product (Michael Dimock, Thermo Trilogy, Corp., pers. comm.). Thus, the number of WDG nematodes applied near the beginning of the product's shelf life can be greater than the number applied at a later date.

Under field conditions, we measured only the efficacy of relatively fresh WDG. In the laboratory we observed decreased nematode viability when the WDG formulation was stored 25 days, but we did not detect any reduced virulence in the nematodes that survived. Therefore, as long as the loss in viability over time is small, field performance of stored WDG is not likely to be reduced. Further experimentation under field conditions will be required to verify this hypothesis. When making comparisons among formulations, all users should be aware that viability and virulence of commercially produced nematodes can vary greatly according to batch (Ricci et al., 1996; Shapiro and McCoy, unpubl.); therefore, the performance of each formulation can be expected to differ on occasion.

Regardless of formulation, application of *S. riobrave* did not result in high levels (e.g., >80%) of *D. abbreviatus* mortality under field conditions. Thus far, >80% larval control has been achieved only using rates of >100 IJs/cm² (Bullock et al., 1999b; Duncan and McCoy, 1996; Duncan et al., 1996, 1999). Using potted citrus, we observed >80% *D.*

abbreviatus suppression in the greenhouse but not in the field. Despite attempts to preclude desiccation (by irrigating) and exclude certain predators from our pots, the biotic or abiotic factors (Kaya, 1990) that were responsible for the higher *D. abbreviatus* mortality observed in the greenhouse relative to the field are not clear. The actual level of observed mortality in caged larvae in the first field experiment is not necessarily indicative of the level of mortality expected in natural populations of *D. abbreviatus*. The caged larvae were exposed only to nematodes for 1 week, and it is likely that longer exposure would have resulted in higher mortality. Therefore, we can conclude little about the level of suppression our nematode applications would produce on natural field populations of *D. abbreviatus*, and this was not our goal.

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