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Detection of mefenoxam-resistant strains of *Peronospora belbahrii*, the causal agent of basil downy mildew, transmitted through infected seeds.

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1 **Detection of mefenoxam-resistant strains of *Peronospora belbahrii*, the causal agent of basil**
2 **downy mildew, transmitted through infected seeds**

3

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16

17 **Abstract**

18

19 Epidemics of basil downy mildew (DM) incited by *Peronospora belbahrii* have been very severe
20 in Italy since 2013, in part due to the very favorable weather conditions, and losses have occurred
21 in many commercial farms, even after repeated mefenoxam treatments. DM populations from basil
22 plants and seeds, which are associated with failure in downy mildew control under field and
23 greenhouse conditions, have been tested for their sensitivity to mefenoxam. Basil plants were
24 inoculated with a sporangial suspension of seven DM populations and treated, before and after
25 inoculation with the pathogen, with different dosages of mefenoxam: 100 µg/ml, which corresponds
26 to the currently applied field dosage, 200 µg/ml and 1000 µg/ml. Azoxystrobin was used at the field
27 dosage as the chemical control. Three out of four DM populations from seeds and two out of three
28 from basil plants were found to be able to infect basil plants in the presence of 100 µg/ml and 200
29 µg/ml of mefenoxam, while the field dosage of azoxystrobin (186 µg/ml) was found to be
30 completely effective. The sensitive populations of *P. belbahrii* were completely controlled by the
31 field dosage of both chemicals also 14 days after the last treatment. This study provides new
32 information on the potential risk of introducing mefenoxam-resistant *P. belbahrii* inoculum in the
33 field through seeds infected by resistant strains.

34

35 **Keywords:** *Ocimum basilicum*; fungicide resistance; phenylamides; downy mildew; seed-
36 transmissions.

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38

39 **Introduction**

40

41 Basil downy mildew (DM), which is incited by *Peronospora belbahrii* (Belbahri et al. 2005; Thines
42 et al. 2009), is one of the most economically important basil diseases, and it has led to significant
43 yield losses in several countries. In Europe, the disease has been reported in Switzerland, Italy,
44 France and Belgium (Lefort et al. 2003; Coosemans 2004; Garibaldi et al. 2004a; 2005), and it has
45 also been observed in Iran (Khateri et al. 2007), the United States, where it has been reported in
46 several States (Roberts et al. 2009, Wick and Brazee 2009; McGrath 2010), Argentina (Ronco et al.
47 2009), Israel (Cohen et al. 2013) and China (Kong et al. 2015). The disease was first described as
48 *Peronospora* sp. (Hansford 1932) in Uganda.

49 The rapid spread of the pathogen to all basil growing areas has probably been favoured by the fact
50 that the pathogen is seed-transmitted (Garibaldi et al. 2004 b; Farahani-Kofoet et al. 2012), as well
51 as by the shift of seed production to African countries, where the pathogen has been present since
52 many years (Hansford 1932).

53 Several studies were conducted to better understand the etiology of basil downy mildew. Elad et al.
54 (2016) discovered oospores in the symptomatic basil leaves and showed as high temperature
55 apparently did not affect the pathogen survival. However, contaminated seeds are considered the
56 primary inoculum source for basil DM because the pathogen rarely produces oospores (Cohen et al.
57 2013; Wyenandt et al. 2015). Garibaldi et al. (2004b) first found infected seeds by *P. belbahrii* in
58 four out 17 commercial seed samples of basil, showing as 0.017% of infected seeds presumably
59 resulted enough for the introduction and spread of the pathogen into areas where it has not been
60 previously reported. Farahani-Kofoet et al. (2012) report that sporangiophores and sporangia can be
61 easily recovered by washing seeds in 80-90% of commercial seed lots. In addition, the possibility of
62 *P. belbahrii* to survive for several years on seeds further complicates the situation for seed
63 producers and farmers (Farahani-Kofoet et al. 2012). The systemic infection of symptomless basil
64 plants and seeds of basil has also been proved using a classic PCR assay (Farahani-Kofoet et al.
65 2012).

66 Because there are no known cultivars resistant or tolerant to DM, despite active research
67 (Wyenandt et al. 2015; Ben-Naim et al. 2015), the control of basil DM is mainly based on the
68 application of fungicides in the field (Gullino et al. 2009; Mershaa et al. 2012; Gilardi et al. 2013;
69 Homa et al. 2014; Wyenandt et al. 2015). Among the various chemicals registered for use on basil
70 against DM as foliar sprays, mefenoxam, which belongs to the phenylamide family (Schwinn and
71 Staub 1987), has been applied extensively in Italy and elsewhere since 2004, because of its
72 excellent preventive, curative and eradicated activities (Gullino et al. 2009).

73 Field resistance to phenylamides has been reported on a wide range of crops in several countries,
74 and the situation is regularly updated in the FRAC Resistance Survey List (www.frac.info).
75 Resistance was first observed for metalaxyl, the first phenylamide fungicide developed, very shortly
76 after its introduction onto the market (Lebeda and Schwinn 1994). Field resistance of *P. belbahrii* to
77 phenylamides was first observed and reported in Israel in 2013 (Cohen et al., 2013), and later in
78 Italy (Pintore et al. 2016; Garibaldi et al. 2016). Failures in the control of basil DM on farms where
79 mefenoxam was applied for its management in northern Italy have been observed starting in 2013.

80 This study was carried out to document changes in sensitivity to mefenoxam of *P. belbahrii*
81 populations obtained from basil plants and seeds, and in order to understand how such populations
82 can spread.

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Materials and methods

Downy mildew (DM) populations

Starting from 2013, several DM populations have been collected in fields and greenhouses in Piedmont and Liguria, where failure in disease control with mefenoxam had been observed. Three *P. belbahrii* populations obtained from infected plants and four populations isolated from contaminated seeds were selected and have been used in this study. Different batches of seeds were collected in order to assess the level of contamination and to explain the difficulties encountered in DM management.

Two populations of *P. belbahrii* from organically produced basil grown in Piedmont were used as reference populations. A list of the tested *P. belbahrii* populations is reported in Table 1. The different DM populations were maintained on artificially infected basil leaves and stored at -20°C (Lebeda and Urban 2010).

Plant material and experimental conditions

During 2014 and 2015, trials were carried out in growth chambers, where small plastic-houses (90 cm high, 50 cm wide and 70 cm long) were built and kept at temperatures ranging from 20 to 23°C and relative humidity close to 95% in order to maintain favourable environmental conditions for DM development (Garibaldi et al. 2007).

Basil seeds from the highly susceptible cultivar Italiko (Semiorio, Salerno, Italy) were used for pathogen propagation and in the *in planta* bioassays.

Plants were produced from heat-treated seeds (65°C for 10 min), in order to guarantee the absence of contamination from infected seeds yielding 20-25 plants/plots. Plastic pots (1.5-L vol., 12 x 12 cm) contained a steam disinfested (90°C for 30 minutes) mixture of white peat: perlite (80:20 v/v) mix (Turco Silvestro, Albenga, Savona) were used. Four replicates were used for each treatment (1 pot /replicate) in a completely randomized design. The experiments were repeated at least three times for each DM population, under completely controlled environmental conditions. The most representative trials are reported in this manuscript.

Artificial inoculation

116 The DM populations were stored at -20°C and propagated on healthy basil plants of Genovese
117 Gigante type, obtained from cv. Italiko heat treated seeds, 7 days before starting the trials in
118 physically separated growth chambers. Basil leaves showing intensive sporulation of the pathogen
119 were shaken in 100 ml of sterile water containing 2 µl of Tween 20. The sporangia suspension was
120 filtered and diluted to a final concentration that ranged from 6.7×10^5 to 1×10^6 sporangia/ml. The
121 artificial inoculation was carried out through nebulisation using a laboratory spray bottle (10 ml
122 capacity). One ml of suspension was used for each replicate (1ml for each of the 4 pots), 24 h
123 before or after the treatment, according to the protocol reported in Tables 2 and 3.

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125 Products and treatment application

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127 Three different dosages of mefenoxam (Ridomil Gold SL Syngenta Crop Protection 43,88% a.i.)
128 were applied on the dates reported in Tables 2 and 3: 100 µg /ml, which corresponds to the currently
129 applied field dosage, and which was applied according to the manufacturer's instructions, a double
130 dosage (200 µg /ml) and a ten times higher dosage (1000 µg /ml) compared to the field rate. Two
131 treatments were applied at intervals of 7 days. Artificial inoculation was made with the pathogen
132 either 24 hours before the first treatment with mefenoxam (A) or 24 hours after the treatment (B).
133 Azoxystrobin (Ortiva, Syngenta Crop Protection, Italy, 23.2% a. i.), which is labelled and
134 recommended on basil in Italy, was used as a reference chemical control 24 hours before the
135 inoculation.

136 The treatments were made as foliar sprays 15-30 days after sowing, at 800 L ha⁻¹, using a handheld
137 1-L capacity sprayer.

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139 Data collection and analysis

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141 The plants were monitored daily and the evaluation of the percent of infected leaves (disease
142 incidence) and of diseased leaf area affected (disease severity) was made, starting from the
143 appearance of the first DM symptoms, using a disease rating scale. Disease severity was
144 examined visually and calculated using the following formula: $DS = [\sum(n^{\circ} \text{ leaves} \times x \text{ 0-5}) / (\text{total}$
145 $\text{ number of leaves recorded})]$ with x 0-5 corresponding to: 1 = from 1 to 10% (midpoint 5 %)
146 infected leaf area; 2 = from 11 to 25% (midpoint 18%) infected leaf area; 3 = from 26 to 50%
147 (midpoint 38%) infected leaf area; 4 = from 51 to 75% (midpoint 63%) infected leaf area; 5=
148 from 76 to 100% (midpoint 85%) infected leaf area.

149 All the collected data were statistically analysed by means of univariate ANOVA, with SPSS
150 software 22, and the means were spread according to Tukey's test ($p < 0.05$).

151

152 **Results**

153 Artificial inoculation of basil plants with the seven tested *P. belbahrii* populations led to a
154 consistent disease level in the inoculated, untreated control plants ranging from 33.8 to 68.3 %
155 disease incidence at 7 days after the last treatment. Population no. 27 was least aggressive,
156 followed by no. 9, all other populations were highly aggressive (Tables 2 and 3). DM symptoms
157 started 7 to 13 days after the artificial inoculation (data not shown). The reference population no.
158 27 (originating from leaves) failed to infect basil plants treated with mefenoxam at the field dosage
159 (100 $\mu\text{g}/\text{ml}$) even 14 days after the last treatment, it is therefore considered as sensitive to
160 mefenoxam (Table 2). Population no. 20 (from seeds) showed 13.0 % of affected leaves with a
161 sporulating leaf area of 5 % at 7 days after the last application of mefenoxam. No significant
162 differences were recorded by spraying mefenoxam as a preventative measure (24 hours before the
163 artificial inoculation) and curative treatment (24 hours after the artificial inoculation) (Tables 2 and
164 3).

165 Four days after the last treatments with mefenoxam at 100 and 200 $\mu\text{g}/\text{ml}$, basil inoculated with
166 DM populations no.22, showed 30.3% to 24.5% of affected leaves with 6.6-6.8% of sporulated leaf
167 areas, respectively, 24 hours before and after the artificial inoculation (Table 2). Thus, this
168 population is considered as resistant to mefenoxam. A similar resistant response to mefenoxam was
169 observed in *P. belbahrii* populations nos.17, 18 and 19, obtained from seeds (Table 3). Disease
170 incidence and severity of the mefenoxam-treated plants were similar to those of the untreated
171 control. The double concentration of mefenoxam reduced disease incidence and severity of DM
172 populations nos. 9, 17 and 18, and provided statistically similar results to those obtained with
173 azoxystrobin, applied as a chemical control (Tables 2 and 3).

174 Mefenoxam, applied at a rate of 1000 $\mu\text{g}/\text{ml}$, was able to completely control all the DM
175 populations, and thus provided the same results as azoxystrobin used as a chemical control.
176 However, this rate of application, which is ten times the recommended dosage, resulted to be
177 phytotoxic to the basil plants and caused light leaf necrosis.

178

179 **Discussion**

180 Most *P. belbahrii* populations tested in this study, which were obtained from infected plants and
181 from contaminated seed lots of basil, generally showed the same aggressiveness when tested on

182 untreated control basil plants except for no.s 9 and 27 which provided significantly less disease.
183 Spraying basil plants with mefenoxam at 100 and 200 µg /ml, 24 hours before or after the artificial
184 inoculation with the pathogen, did not significantly reduce disease severity and incidence of
185 populations no.s 17, 18, 19 and 22, which are therefore considered as resistant to mefenoxam.
186 Population no. 27 is considered as sensitive, whereas populations no.s 9 and 20 may contain a low
187 proportion of resistant spores. Since in biotrophic pathogens it is rather tricky to produce single
188 sporangium strains, all tested populations must be considered as mixtures of strains with different
189 sensitivities to fungicides. The detection limit of resistant sporangia in strain mixtures is not known
190 for *P. belbahrii* and should be evaluated for the used bioassay procedure in future experiments.
191 Also the definition of resistant strains may vary depending on the authors. Cohen et al. (2013)
192 reported on a resistant *P. belbahrii* population having survived a preventive treatment of potted
193 basil plants with 1,000 µg/ml mefenoxam, while a sensitive population did not cause any symptoms
194 after a spray with 10 µg/ml.

195 Although resistance to mefenoxam in populations of *P. belbahrii* obtained from infected basil plants
196 has already been reported in Israel (Cohen et al. 2013) and in Italy (Pintore et al. 2016; Garibaldi et
197 al. 2016), this is the first report on resistant populations of isolates of *P. belbahrii* originating from
198 seeds. Various methods are available to assess the sensitivity of downy mildew to fungicides
199 (Urban and Lebeda 2006). In the present study, a bioassay with whole plants has been used
200 comparing the sensitivity to mefenoxam of DM isolates collected in field from basil plants and DM
201 isolates from seedling obtained from contaminated seeds.

202 *P. belbahrii* was first reported in northern Italy at the beginning of 2003 (Garibaldi et al. 2004a),
203 and later spread to other Italian production areas (Garibaldi et al. 2004b). Since its introduction, the
204 control of this pathogen has primarily been dependent on the application of thiram and propamocab,
205 which have only shown moderate efficacy. In previous research, it was found that mefenoxam was
206 the most active fungicide for DM control, and a label extension, based on directive 91/414/CE, was
207 therefore immediately requested and obtained (Gullino et al. 2009). Most basil growers applied the
208 manufacturers' recommended rates and generally applied mefenoxam once per crop cycle,
209 alternating with other fungicides with different modes of action, such as azoxystrobin, which
210 belongs to the Quinone outside inhibitor (QoI) group, mandipropamid, which belong to the
211 carboxylic acid amide CAA group, and fluopicolide, which belongs to the benzamide group. As a
212 general rule for other downy mildews, those chemicals should be applied preventatively or as early
213 as possible in the disease cycle, in a limited number of sprays in order to avoid the selection of
214 resistant strains (Gisi and Sierotzki 2008; Hermann and Gisi, 2012; MacBean 2012).

215 Resistance to phenylamides emerged rather quickly after their introduction in many oomycetes on
216 vegetable crops such as *Pseudoperonospora cubensis* (Reuveni et al. 1980; Katan and Bashi 1981),
217 *Peronospora tabacina* (Bruck et al. 1982) and *Bremia lactucae* (Crute et al. 1987).
218 Even though mefenoxam is marketed for basil treatments in a mixture with copper oxychloride in
219 Italy, growers have observed a reduced efficacy of this fungicide and yield losses since 2013.
220 Seeds are generally recognised as the main source from which *P. belbahrii* survives from season to
221 season, because the pathogen very rarely produces oospores (Cohen et al. 2013; Wyenandt et al.
222 2015). This study confirms the presence of resistant field populations of DM of basil, and also
223 provides evidence that basil seeds are a potential source of mefenoxam-resistant inoculum of *P.*
224 *belbahrii*. In a previous study, Thomas et al. (2014) found isolates of *Didymella bryoniae* from two
225 seed lots resistant to thiophanate-methyl, which is commonly used for the management of
226 watermelon gummy stem blight. The aggressiveness showed by DM populations of isolates from
227 seeds associated with mefenoxam resistance suggested a notable ability to compete with sensitive
228 DM populations highlighting a high risk of spread in field. However, specific studies are needed to
229 investigate the fitness of these isolates. Our results suggest the need for anti-resistance strategies for
230 the management of DM, not only in the field but also for seed production as well as for seed
231 dressing. Considering the high probability of using seed lots already infected, seed dressing with
232 fungicides with different mode of action of mefenoxam, should represent the first preventative
233 strategy to be consider for seed producers and farmers. However, among non-chemical treatments
234 of basil seeds with hot air (65°C for 10 min), and thyme oil may be suggested (Gilardi et al. 2015).
235 Moreover, the presence of mefenoxam resistant *P. belbahrii* populations in basil production areas in
236 Italy requires a continuous sensitivity monitoring of populations in fields as well as from seeds.

237

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244

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346 Table 1 List of the populations and their origins from basil leaves and from contaminated seeds

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Code	Origin of samples/seed company	Samples
9	Castagnole, Piedmont (Northern Italy)	Basil leaves
22	Compagnia del basilico, Liguria (Northern Italy)	Basil leaves
27	Nichelino, Piedmont (Northern Italy)	Basil leaves
17	Furia, Piedmont ((Northern Italy)	Basil Seeds
18	Semiorto, Liguria (Northern Italy)	Basil Seeds
19	SAIS, Liguria (Northern Italy)	Basil Seeds
20	Franchi sementi, Piedmont (Northern Italy)	Basil Seeds

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365 Table 2 Disease incidence (% of infected leaves) and Disease severity (% of affected leaf area) on basil plants (cv
 366 Italiko) caused by *Peronospora belbahrii* populations obtained from basil plants 4 , 7, and 14 days after the last
 367 treatments

Treatments	Dosage a.i (µg /ml) and time of application	Disease incidence caused by DM populations n								
		22			9			27		
		Dat 4 ^y	Dat7	Dat 4	Dat7	Dat 4	Dat7	Dat 4	Dat7	Dat 14
Non inoculated control	-	0.0 a ^z	0.0 a	0.1 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	1.3 a
Untreated control	-	53.8 c	64.8 c	36.1 d	45.3 c	16.4 B	33.8 b	36.2 b		
Mefenoxam	100 A ^x	30.3 bc	35.7 b	16.8 c	24.7 b	0.0 a	0.0 a	0.0 a		
Mefenoxam	200 A	24.5 b	34.9 b	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a		
Mefenoxam	1000 A	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a		
Mefenoxam	100 B	30.5 bc	47.1 bc	13.3 bc	33.0 bc	0.0 a	0.0 a	0.0 a		
Azoxystrobin	186 A	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a		

Treatments	Dosage a.i. (µg /ml) and time of application	Disease severity (0-100) caused by DM populations n								
		22			9			27		
		Dat 4	Dat7	Dat 4	Dat7	Dat 4	Dat7	Dat 4	Dat7	Dat 14
Non inoculated control	-	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	1.0 a	
Untreated control	-	23.4 b	24.0 c	9.9 b	14.3 b	3.0 b	5.0 b	6.1 b		
Mefenoxam	100 A	6.4 a	11.4 b	4.3 ab	8.9 b	0.0 a	0.0 a	0.0 a		
Mefenoxam	200 A	6.8 a	8.3 ab	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a		
Mefenoxam	1000 A	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a		
Mefenoxam	100 B	9.1 a	12.7 b	6.1 b	9.9 b	0.0 a	0.0 a	0.0 a		
Azoxystrobin	186 A	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a		

368 ^xTime of application: A, 24h before the artificial inoculation of the pathogen. B, 24h after the artificial inoculation of
 369 the pathogen

370 ^y Days after the last treatment

371 ^z Values with the same letter in the same column are not significantly different, according to Tukey's Test (p<0.05)

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373 Table 3 Disease incidence (% of infected leaves) and Disease severity (% of affected leaf area) on basil plants (cv
 374 Italiko) caused by *Peronospora belbahrii* populations obtained from basil seeds 4 and 7 days after the last treatments

Treatments	Dosage a.i. (µg /ml) and time of application	Disease incidence caused by DM populations n												
		17		18		19		20						
		Dat 4 ^y	Dat7	Dat 4	Dat7	Dat 4	Dat7	Dat 4	Dat7	Dat 4	Dat7			
Non inoculated control	-	0.0	0.0	0.0	0.0	0.0	0.0	a	0.0	a	0.0	a	0.0	a
Untreated control	-	54.7	68.3	55.1	61.7	51.7	60.0	bc	60.0	b	46.0	b	54.3	b
Mefenoxam	100 A ^x	23.1	38.5	33.2	47.0	57.9	64.2	c	64.2	b	5.3	a	13.0	a
Mefenoxam	200 A	0.0	18.7	18.4	27.1	40.6	52.4	ab	52.4	b	0.0	a	0.0	a
Mefenoxam	1000 A	0.0	0.0	0.0	2.3	0.0	0.0	a	0.0	a	0.0	a	0.0	a
Mefenoxam	100 B ^b	18.5	37.1	34.2	39.1	40.3	51.1	bc	51.1	b	0.0	a	0.0	a
Azoxystrobin	186 A	0.0	0.0	0.0	0.0	0.0	0.0	a	0.0	a	0.0	a	0.0	a
Treatments	Dosage a.i. (µg /ml) and time of application	Disease severity (0-100) caused by DM populations n												
		17		8		19		20						
		Dat 4	Dat7	Dat 4	Dat7	Dat 4	Dat7	Dat 4	Dat7	Dat 4	Dat7			
Non inoculated control	-	0.0	0.0	0.0	0.0	0.0	0.0	a	0.0	a	0.0	a	0.0	a
Untreated control	-	23.0	34.6	20.5	26.8	17.8	22.7	cd	22.7	c	24.3	b	27.2	b
Mefenoxam	100 A	4.8	12.9	9.2	13.5	18.7	20.8	ab	20.8	c	3.0	a	5.0	a
Mefenoxam	200 A	0.0	2.8	3.2	6.8	12.6	18.7	a	12.6	bc	0.0	a	0.0	a
Mefenoxam	1000 A	0.0	0.0	0.0	0.5	0.0	0.0	a	0.0	a	0.0	a	0.0	a
Mefenoxam	100 B	4.4	15.1	8.3	12.8	8.3	13.0	ab	8.3	b	0.0	a	0.0	a
Azoxystrobin	186 A	0.0	0.0	0.0	0.0	0.0	0.0	a	0.0	a	0.0	a	0.0	a

375 ^x Time of application: A, 24h before the artificial inoculation of the pathogen. B, 24h after the artificial inoculation of
 376 the pathogen

377 ^y Days after the last treatment

378 ^z Values with the same letter in the same column are not significantly different, according to Tukey's Test (p<0.05)

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