

Factors Influencing Infection of Onion Leaves by *Alternaria porri* and Subsequent Lesion Expansion

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ABSTRACT

Everts, K. L., and Lacy, M. L. 1996. Factors influencing infection of onion leaves by *Alternaria porri* and subsequent lesion expansion. Plant Dis. 80:276-280.

Alternaria porri conidia deposited on onion leaves formed one to several germination tubes and appressoria and often penetrated at more than one locus under conditions favorable for infection. After 3 h in the dew chamber at 24°C following inoculation of onion leaves, 73% of conidia had germinated and 5% had formed appressoria. Infection hyphae were not observed until 6 h following inoculation, at which time 2% of conidia had formed infection hyphae and 0.5% of conidia had caused visible lesions. Length of dew period was significantly positively correlated with lesion numbers ($r = 0.784$) but not with lesion size. There were two types of lesions: expanding and nonexpanding (flecks of ≤ 2 mm in diameter). Expanding lesions resulted even when plants inoculated with dry conidia of *A. porri* were incubated in a growth chamber under conditions not conducive to infection for 4 days prior to being placed in a dew chamber for 24 h under conditions conducive to infection, indicating that conidia survived well under these conditions. Flecks formed concurrently with expanding lesions. Germinated conidia were inevitably found near the centers of flecks, but, for unknown reasons, these flecks did not continue to expand.

Additional keywords: *Allium cepa*, purple blotch

Onion purple blotch disease, caused by *Alternaria porri* (Ellis) Cif., causes serious losses in onion (*Allium cepa* L.) crops wherever onions are grown (2,13-15). Yield losses occur primarily through loss of leaf tissue and subsequent reduction in rate of bulb development (15). Losses of up to 50 (3) and 100% (17) have been reported. The severity of the disease in South Africa recently led to examination of potential seed treatments as a partial control (4).

The events in the establishment of a purple blotch epidemic include conidial germination, germ tube elongation, penetration, lesion initiation, and lesion expansion. These events are influenced by environmental factors including dew duration, relative humidity, and temperature. Early investigations of the germination process of *A. porri* established the relation of increasing temperature to increasing rate of conidial germination and germ tube growth (3,17). The optimum temperature was reported to be as high as 36°C for

germ tube formation (3), was 20 to 25°C for appressorial formation, and was 22 to 30°C for hyphal growth (6).

Environmental conditions before spore deposition also influenced the ability of conidia to germinate. Previously (8), we found that the ability of conidia to germinate increased as the dew period increased inside a dew chamber (99 to 100% relative humidity [RH]) in which the conidia formed. For example, 26% of conidia formed during 9 h of dew germinated, while 96% of those formed during 21 h of dew germinated.

Conidia of *A. porri* can produce multiple germ tubes that grow across the leaf surface before forming appressoria (11). An appressorium may form over a stomate or on the epidermis remote from stomata where direct penetration occurs. After direct or indirect penetration, mycelia grow subcuticularly and ramify inter- and intracellularly (3).

Temperature influenced lesion formation, and an optimum range (20 to 25°C) for lesion initiation was reported (6). Increasing humidity levels and duration of high humidity periods resulted in an increase in the number of expanding lesions (purple blotches); however, under varying humidity levels (20 to 100%) some flecks (0.5- to 2-mm² white irregular spots) that developed did not expand (6). Serial sections of white flecks showed vacuolated dead epidermal and palisade cells, but no mycelia (6). The duration of dew during conidial formation also influenced fleck

formation and lesion expansion. Previously (8), we found that conidia formed during 12 h dew caused only flecks; however, conidia formed during 16 h dew formed both flecks and typical lesions. We speculated that flecks may have resulted when conidia causing their formation were immature and not aggressive enough to establish a lesion before encountering a plant resistance response.

As in many diseases caused by *Alternaria* spp., the physiological age of onion tissue influences the extent of damage caused by a lesion (14,18) and the amount and timing of leaf tissue loss influences yield (15). Onion purple blotch damage increased as leaf age increased and as the entire plant aged (14). Older tissue is also more susceptible to *Alternaria* pathogens than is young tissue in potato, eggplant, cabbage, carrot, wheat, and other crops (18,19).

The fact that increasing disease levels frequently occur late in the growing season (9,13,14) is often attributed only to increasing age and susceptibility of plant tissue. However, increasing numbers of lesions were found on potted onion trap plants placed in the field weekly that were uniform in age at each week of sampling (9). Other factors such as increasing conidial numbers, and weather that favors spore deposition, infection, and lesion development, may also affect the increasing level of disease observed late in the season (9,13).

Information on the effect of dew period, temperature, and humidity on spore development, infection, and lesion formation is used in formulating control strategies for purple blotch (2,9,13). However, information on the effect of length of dew period on lesion formation following spore deposition is lacking. Lack of correlation between periods of high conidial release and subsequent lesion formation on trap plants in our previous work (6) indicated that conditions favoring spore formation and release may have differed from conditions favoring lesion development. Thus, information on the influence of dew period on lesion development is needed.

The following studies were undertaken to examine (i) the effect of dew period on the ability of conidia to germinate and subsequently infect onion leaves, (ii) the ability of conidia to survive in the absence of moisture prior to infecting onion leaves, and (iii) some factors influencing lesion expansion.

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Accepted for publication 16 November 1995.

Publication no. D-1995-1222-05R
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MATERIALS AND METHODS

Production of conidia. Highly virulent *A. porri* isolates AAP-1 (obtained from Marvin Miller, Texas A&M University) or AAP-3 (isolated from Michigan-grown onions in 1986) were used in all experiments. To prepare the fungus for long-term storage, it was grown on V8-agar plates (16) for 6 days, and a 6-mm disk of colonized agar was transferred to sterilized muck soil in test tubes, incubated for 6 days at 25°C (shaken daily), and then stored at 5°C.

Alternaria porri isolates AAP-1 and AAP-3 sporulated on V8-juice agar (16); however, sporulation was stimulated by exposing colonies to near-ultraviolet (NUV) light (peak wavelength 360 nm) (5, 10, 20). For conidial production, 0.1 to 0.2 g of infested muck soil from soil tubes was placed on sterile V8 agar in 9-cm-diameter petri plates. Plates were incubated at room temperature in darkness for 6 to 8 days, exposed to NUV light at a distance of 0.5 m for 24 h to initiate conidiophore formation (5), and placed in the dark for 1 to 3 additional days. Conidia were produced abundantly using this system.

To harvest conidia, sterile water was poured over *A. porri* colonies on V8-agar plates and conidia were suspended by lightly rubbing the colony surface with a sterile glass rod. Conidia were collected from the resulting spore suspension on a 0.8- μ m-pore-size membrane filter (Millipore Filter Corp. Bedford, MA) and then dried for 24 h over anhydrous CaSO_4 before use.

Inoculation. Onion plants of cv. Spartan Banner 80, a yellow-skinned long-day storage onion, were used in all experiments. Dry conidia were weighed on glassine weighing papers, then dispersed over onion plants positioned in a settling tower by gently blowing them off with puffs of air from a Pasteur pipette. The settling tower consisted of a galvanized sheet metal cylinder (61 cm diameter by 77 cm deep) mounted on a wooden base. Plants were placed at the base of the settling tower on a custom-built revolving platform (5 to 6 rpm) to allow more uniform deposition and expose greater leaf surface area to settling spores. A metal cover was placed over the tower after conidia were dispersed and spores were allowed to settle on the plants for 10 min. The area of the base of the settling tower was 2,922 cm^2 . There were approximately 1.1×10^9 and 2.2×10^9 conidia in 2.5 and 5.0 mg of conidia, respectively, providing concentrations of approximately 3,750 and 7,500 conidia per cm^2 of base area. In preliminary experiments onion plants free from purple blotch symptoms were grown from dormant bulbs in the greenhouse for 5 weeks, inoculated with 2.5 mg of conidia of isolates AAP-1 or AAP-3, subsequently placed in a 24°C dew chamber for 24 h, then moved to the growth chamber

for 4 days at 24°C. A mean of 26 (isolate AAP-1) and 64 (isolate AAP-3) expanding lesions and flecks (combined) developed per plant.

Germination, appressoria, infection hyphae, and lesions. Onion plants free from purple blotch symptoms were grown from 5.0- to 6.5-cm-diameter dormant bulbs in the greenhouse for 5 weeks, and plants were then placed in the settling tower where they were inoculated with 5.0 mg of *A. porri* conidia as described above. Twenty plants were then placed in the dew chamber at 24°C. After 3, 6, 12, 18, or 24 h of dew, four randomly selected plants were removed after each period and leaves were sampled. Plants were completely randomized within the settling tower, dew chamber, and growth chamber. Sections of leaves were removed from the outermost full-sized leaf of each plant, cut lengthwise, flattened, and either used fresh or placed in fixative solution of a 1:1 mixture of absolute ethanol/glacial acetic acid for 24 h to clear the tissue. Fixed or fresh tissue pieces were mounted on slides and stained with 0.1% cotton blue in 85% lactic acid, and at least 150 conidia from each dew period were examined under a light microscope (200 \times and 400 \times) for the presence of germ tubes, appressoria, infection hyphae, or for lesion formation. The experiment was repeated once with similar results.

Germination and appressoria formation of conidia were also studied using a scanning electron microscope. Onion plants were inoculated with 2.5 mg of *A. porri* conidia in the settling tower and placed in a dew chamber in darkness at 24°C. Leaf pieces were excised after 3 or 6 h and vapor-fixed with OsO_4 for 96 h in a closed chamber, air dried for 2 days and then mounted, coated, and examined in a JEOL-JSM-35 CF scanning electron microscope.

Penetration. Five-week-old onion plants grown from dormant bulbs in the greenhouse were inoculated with 5.0 mg of *A. porri* conidia in the settling tower as previously described. Plants were then placed in the dew chamber for 48 h at 24°C. The outermost full-sized leaf was sampled by fixing and mounting the tissue as described above. Each fixed and stained leaf was examined microscopically to determine the percentage of conidia that penetrated directly or through stomates. The experiment was repeated once with similar results.

Dew period. Four-week-old plants grown from dormant bulbs in the greenhouse were inoculated with 2.5 mg of *A. porri* conidia in the settling tower. Forty-two plants were placed in the dew chamber at 23°C. Six randomly selected plants were removed after 6, 12, 18, 24, 30, or 42 h, respectively, and placed in the growth chamber at 23°C (36 to 48% RH) with a 12-h day length. Plants were incubated in

the growth chamber following removal from the dew chamber, and were examined 7 days following inoculation (which included the variable dew periods plus the subsequent periods of incubation in the growth chamber). Plants were completely randomized in the growth chamber. Lesion numbers were counted and lesion size was measured. The experiment was repeated once with similar results. Simple correlations between length of dew period and lesion number or lesion size, and between lesion number and lesion size were determined with MSTAT statistical package (Department of Crop and Soil Sciences, Michigan State University, East Lansing).

Flecks. Six onion plants were inoculated as described above and placed in a dew chamber in darkness at 24°C for 24 h, then plants were moved to a 24°C growth chamber. Leaves were examined visually 3 days after inoculation and all lesions/flecks were circled with a permanent marker (at this stage it was impossible to tell flecks from lesions). Plants were replaced in the dew chamber, circled lesions were examined again 8 days after inoculation, and numbers of lesions that expanded beyond fleck size (2 mm diameter) were recorded. Fresh leaf segments (approximately 1.5 \times 1.5 cm) were flattened and mounted whole on glass slides, 2 to 3 drops of stain (0.1% cotton blue in 85% lactic acid) were applied, and a coverslip was affixed and weighted down with a lead weight. Flecks were examined under a microscope (100 \times or 400 \times) 1 h after staining to determine whether germinating conidia were present on the flecks and, if so, whether penetration of the epidermis had occurred. The experiment was repeated once with similar results.

Conidial survival. To determine the ability of *A. porri* conidia to survive on leaf surfaces after deposition, outermost leaves of 20 4-week-old onion plants were removed so that three or four leaves remained per plant. These plants were then inoculated with 3.0 mg of conidia of isolate AAP-3 in the settling tower as described above. After inoculation, four plants were immediately placed in the dew chamber, and the remaining plants were placed in the growth chamber (24°C and 36 to 48% RH). After 24 h in the dew chamber, the first four plants were moved to the growth chamber. Four different plants were removed from the growth chamber and placed in the dew chamber. This process was repeated so that groups of four plants had 0, 1, 2, 3, or 4 days exposure to dry conditions not conducive to infection before being placed in the dew chamber where infection could occur. Plants were completely randomized in the settling tower and growth chamber. Expanding lesions (those that quickly became >2 mm in diameter and continued to enlarge) and nonexpanding lesions (flecks) that remained \leq 2 mm in diameter formed

concurrently on inoculated leaves. Expanding lesions and flecks were counted 72 h after plants were placed in the growth chamber following removal from the dew chamber. The experiment was repeated once with similar results. To determine treatment effects, analyses of variance were conducted using the MSTAT statistical package.

RESULTS

After 3, 6, 12, or 24 h of dew, 73, 84, 84, and 90%, respectively, of *A. porri* conidia on onion leaf surfaces had germinated; 5, 34, 44, and 63%, respectively, had formed appressoria; and 0, 2, 13, and 23%, respectively, had formed infection hyphae (Fig. 1). No visible lesions formed after 3 h of dew. However, 0.5, 6, and 15% of conidia had caused visible lesions (flecks and expanding lesions) after 6, 12, and 24 h of dew, respectively.

Penetration. Germ tubes from individual multicellular conidia had germinated and could penetrate through stomata after as little as 3 h of dew (Fig. 2). The conidium shown in Figure 2 is penetrating a stomate. Conidia formed one to several germ tubes and appressoria and could penetrate leaves directly after as little as 6 h of dew (Fig. 3 A,B). Germ tube growth was more extensive after a 6-h dew period. Although most germ tubes had not formed appressoria after 6 h of dew, a few appressoria could be observed (Fig. 3A,B). Conidia were capable of causing penetrations at more than one locus. Forty percent of conidia examined had germ tubes that penetrated through stomata and 69% had germ tubes that penetrated directly. Germ tubes from individual conidia could penetrate either through stomates, or directly, or both. Usually one or two germ tubes per conidium penetrated the leaf.

Dew period. Lesion numbers (expanding lesions and flecks) on leaves increased with increasing length of dew period at 23°C (Fig. 4). There was a significant ($P =$

<0.01) positive correlation ($r = 0.784$) between length of dew period and lesion numbers. Length of dew period ($r = -0.213$) and lesion numbers per plant ($r = -0.154$) were not significantly correlated with lesion size.

Flecks. Of 77 initial infection points that remained flecks or later became lesions that were circled with a permanent marker 3 days after inoculation, 12 had expanded to >2 mm in diameter by 8 days after inoculation (expanding lesions) while 65 remained unchanged (flecks). Of 25 flecks mounted and examined, all had one or more germinated conidia at or near the center of the fleck and at least one of the germ tubes had penetrated the leaf, either directly by means of an appressorium or

through a stomate. In several expanding lesions examined, there was widespread proliferation of mycelia in the senescent or dead tissue; in the flecks, there was no evidence of continued proliferation of mycelia, even though penetration had occurred.

Conidial survival. Expanding lesions >2 mm in diameter were observed even when plants were kept in the growth chamber for 1 to 4 days following inoculation before being placed in the dew chamber (Fig. 5). An average of 5.5, 3.5, 2.3, 1.8, and 1.5 expanding lesions per plant formed after plants were incubated in the growth chamber for 0, 1, 2, 3, or 4 days, respectively, prior to a dew period. However, because of the high variability in



Fig. 2. Conidium of *Alternaria porri* penetrating through a stomate of onion cv. Spartan Banner 80 following 3 h of dew (bar = 10 μ m).

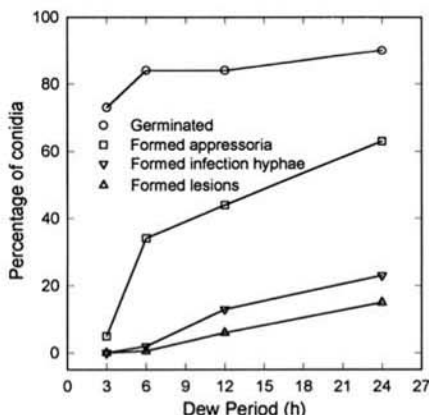


Fig. 1. Percentage of total *Alternaria porri* conidia to germinate, form appressoria or infection hyphae, and cause visible lesions on onion cv. Spartan Banner 80 leaves after variable dew periods.

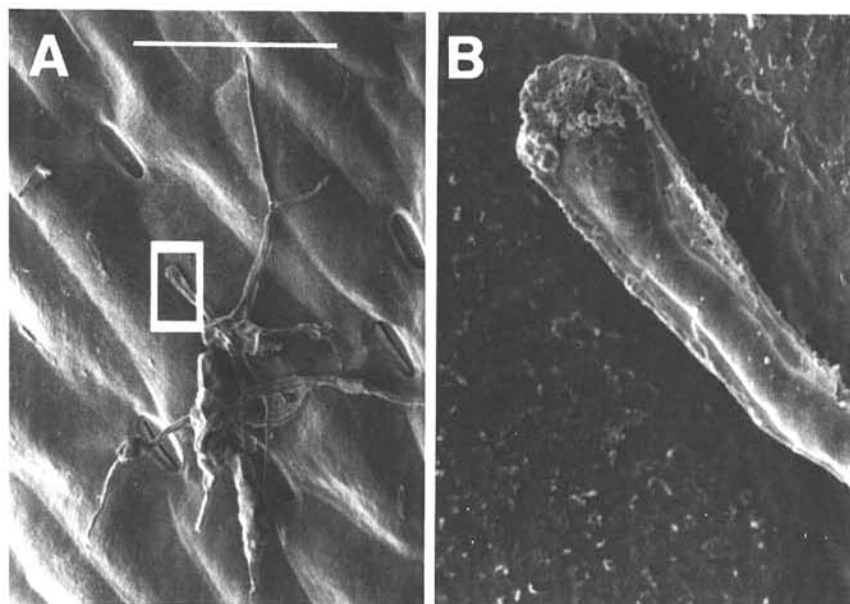


Fig. 3. (A) Germinating conidia of *Alternaria porri* on onion cv. Spartan Banner 80 with an appressorium (bar = 100 μ m) and (B) with appressorium further magnified 10 \times .

lesion numbers per plant, none of these numbers was significantly different from any other, even though the trend toward lower lesion numbers was evident. Mean numbers of flecks (nonexpanding lesions ≤ 2 mm in diameter) remained relatively constant whether plants were placed in the dew chamber immediately after inoculation or incubated for 4 days before being placed in the dew chamber. Flecks were found on inoculated plants but not on noninoculated controls, and one to several spores were observed at the center of each fleck.

DISCUSSION

In previous studies (8,11) the infection process in onion purple blotch was influenced by environmental events that occurred before conidial dissemination because of environmental influence on conidial maturation. For example, at RH $< 81\%$, conidia that formed had fewer cells than conidia formed at 100% RH (11). The length of dew period during conidial formation affected germinability, and as dew period duration in which conidia developed increased from 12 to 20 h, the number of lesions that developed after inoculation increased.

Once conidia develop and are disseminated, conditions will again affect the ability of *A. porri* to survive until the environment favors germination, germ tube growth, infection, and lesion and fleck formation. Fahim and El-Shehedi (11) demonstrated that amount of germination was not inhibited by sunlight; however, germ tube elongation was reduced. Low RH or alternating wet-dry conditions may affect conidial survival subsequent to germination in *Alternaria* spp. Bock (6) found that a 6-day postinfection period in which RH was $< 80\%$ favored fleck formation over lesion formation. Studies of tobacco brown spot caused by *A. alternata* demonstrated that total hours of leaf wetness

(either continuous or interrupted) determined lesion numbers and that a post-inoculation dry period from 1 h to 20 days caused decreasing numbers of lesions (21). The decrease was most dramatic after 10 days. The authors also noted that when the postinoculation dry period exceeded 10 days, no pinpoint lesions, which may be similar in origin to flecks on onion plants, were observed. While we also saw a trend toward decreasing numbers of lesions following a postinoculation dry period, no obvious pattern regarding fleck formation was found. However, fleck formation might have decreased if the dry period prior to dew had been lengthened beyond 4 days.

Despite the many environmental factors that can influence *A. porri* conidial formation, dissemination, and survival, thousands of conidia may be present in a cubic meter of air during peak times of dissemination and settle on onion plants (9). Even if the proportion that survives is low, some conidia survive to infect. Subsequent to deposition, the proportion of infections that form expanding lesions may be low. Under laboratory conditions the proportion of expanding lesions formed from total infections (flecks + lesions) was approximately 15%.

Once conditions favor infection, the development of germ tubes, appressoria, infection hyphae, and resulting lesions progresses relatively rapidly in purple blotch disease of onion. We demonstrated here that *A. porri* conidia penetrate and form lesions after as little as 6 h of dew. In other *Alternaria* spp. lesion formation also progresses very rapidly. Lesion formation following 2 or 4 h of leaf wetness was demonstrated for *A. cucumerina* on muskmelon (7), 4 h for *A. alternata* on tobacco (21), and 9 h (the shortest dew period tested) for *A. helianthi* on sunflower (1). Utilizing an infection model developed for *Alternaria* blotch on apple, Filajdić and Sutton (12) calculated that 5.1 h of leaf

wetness would lead to light (0.2%) leaf area infection. Because conidia are able to survive after dissemination on onion leaves, the onset of dew may result in formation of lesions even if there have not been conditions conducive to sporulation for several days. We previously found that conidial concentration of *A. porri* in air above an onion field in Michigan was not well correlated with development of lesions on onion trap plants (9). This may have occurred in part because previously disseminated spores remained viable until conditions favored germination and infection, or because conidia may have been incompletely liberated during conditions favorable for infection. In any event, correlation of the number of conidia released to subsequent lesion formation may be improved if additional equations could be included in a prediction model. These equations might account for (i) conditions prior to release and their effect on conidial maturation, (ii) conidial release on previous days, (iii) survival of conidia subsequent to dispersal, and (iv) conidial germination, appressorial formation, and infection following variable dew periods. This information is potentially valuable for growers who currently apply fungicides on a calendar basis.

Previous reports indicated that flecks of *A. porri* were vacuolated but no mycelia were visible, and that periods of low RH during infection and postinfection periods favored flecks (4). We have demonstrated that penetration occurs in flecks, but mycelial proliferation in leaf tissue does not occur. We also found that flecks formed concurrently with lesions. The occurrence of germ tube penetration in flecks disproved our previous hypothesis (8) that flecks caused by *A. porri* might be the plant's response to the presence of the pathogen on the leaf surface rather than to germ tube penetration.

ACKNOWLEDGMENTS

Support of this research by the Michigan Agricultural Experiment Station and the Michigan Onion Research Committee is gratefully acknowledged.

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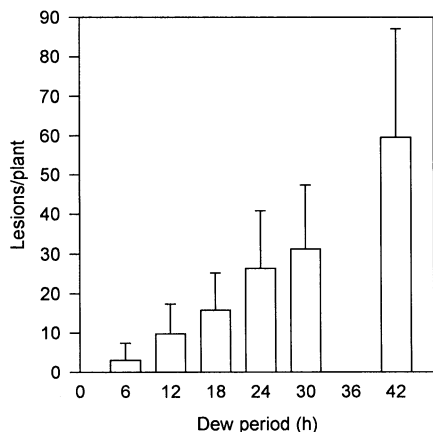


Fig. 4. Influence of dew period duration on numbers of lesions (expanding lesions and flecks) on onion cv. Spartan Banner 80 caused by *Alternaria porri*. Error bars represent the standard error of the mean.

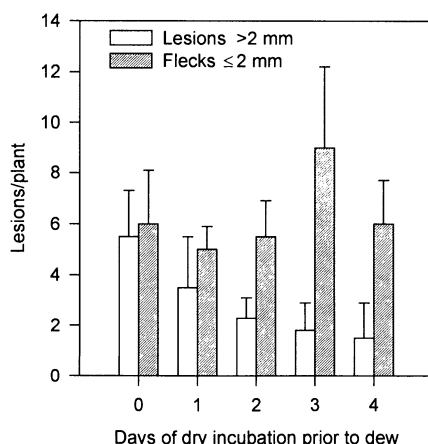


Fig. 5. Infection of onion cv. Spartan Banner 80 leaves by *Alternaria porri* conidia subjected to variable dry periods prior to 24 h of dew. Error bars represent the standard error of the mean.

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