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Effects of Commercial Fatty Acids on Cutinase Release by *Ascochyta pisi*

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With 7 figures

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Abstract

The effect of a range of commercially available C_{16} and C_{18} fatty acids, on the release of cutinase by *Ascochyta pisi*, was studied. When juniperic acid was used as sole carbon source, cutinase activity was released in the culture medium, and was markedly enhanced by post-treatment of *A. pisi* cultures with acetone used as enzyme extractor. Without acetone, less cutinase was naturally released in culture medium containing juniperic acid at 0.5 %, than at 0.01 % or 0.05 %. Upon post-treatment with acetone, the same level of cutinase was released with all three concentrations, thus suggesting that the enzyme was induced, but not completely released in the presence of 0.5 % juniperic acid. When ricinelaidic or ricinoleic acids were supplemented at 0.5 % to cutin in the culture medium, they strongly inhibited the release of cutinase, even with acetone post-treatment. Comparable inhibition by ricinoleic acid was also observed when juniperic acid was used as cutinase inducer, thus suggesting that not only the release, but also the production of cutinase were inhibited.

Zusammenfassung

Einflüsse von käuflichen Fettsäuren auf die Cutinasefreisetzung durch *Ascochyta pisi*

Untersucht wurde der Einfluß von käuflichen C_{16}- und C_{18}-Fettsäuren auf die Freisetzung von Cutinase durch *Ascochyta pisi.* Wenn Junipersäure als einzige Kohlenstoffquelle vorhanden war, wurde Cutinase in das Kulturmedium freigesetzt, und dies wurde durch eine Nachbehandlung der *A. pisi*-Kulturen mit Aceton, als Enzymextraktor, stark erhöht. Ohne die Acetonbehandlung wurde weniger Cutinase in das Kulturmedium freigesetzt, wenn Junipersäure in einer Konzentration von 0,5 % statt 0,01 % oder 0,05 % vorhanden war. Nach einer Acetonnachbehandlung jedoch wurde die gleiche Cutinasemenge bei allen Konzentrationen freigesetzt, was darauf hindeutet, daß das Enzym induziert, aber im Vorhandensein von 0,5 % Junipersäure nicht vollständig freigesetzt war.

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Wenn 0,5 % Ricinelaid- oder Ricinolsäure Cutin im Kulturmedium beigemischt wurde, wurde die Freisetzung von Cutinase stark gehemmt, auch nach einer Acetonnachbehandlung. Auch wenn Junipersäure als Cutinaseinduzierer verwendet wurde, wurde eine gleiche, durch Ricinolsäure verursachte Hemmung beobachtet. Dies deutet darauf hin, daß nicht nur die Freisetzung, sondern auch die Produktion von Cutinase gehemmt wurde.

Cutinase is an esterase able to hydrolyze cutin, a polymer of C_{16} and C_{18} fatty acids which is the main component of plant cuticle. Cutinase is produced by some fungi during their penetration in the host-plant. The most important works concerning this subject were summarized by KOLATTUKUDY (1985) and KÖLLER (1991).

Cutinase is known to be induced in fungi by using cutin as sole source of carbon. Fungal cutinase was also induced by low concentrations of fatty acids extracted from hydrolyzed cutin (LIN and KOLATTUKUDY 1978, WOLOSHUK and KOLATTUKUDY 1986, PODILA *et al.* 1988); its activity was severely inhibited by reagents directed towards active serine, such as diisopropyl fluorophosphate (DFP) (PURDY and KOLATTUKUDY 1975).

In a previous paper (NASRAOUI *et al.* 1990), we have shown that two pea pathogens, *Mycosphaerella pinodes* (Berk. & Blox.) Vestergr. [*Ascochyta pinodes* Jones], and *Ascochyta pisi* Lib., released cutinase activity when grown in a medium containing cutin as sole source of carbon; this cutinase was involved in the successful infection of the host.

In the present work, we studied the effects of some commercial C_{16} and C_{18} fatty acids on *in vitro* release of cutinase by *A. pisi.*

Materials and Methods

Growth conditions

Spore suspensions of *A. pisi* (strain LG, kindly donated by Dr SPIRE, INRA, France) were submersed in the culture media (described in *Cutinase induction* paragraph) in Erlenmeyer flasks, to reach a final volume of 10 ml containing 5×10^5 spores/ml. The vials were incubated on a rotary shaker at 26 °C, with a photoperiod of 16 h light.

For cutinase activity measurement, *A. pisi* cultures of 15 days were filtered; filtrates were collected and mycelium was resuspended in fresh mineral solution of a modified Czapeck-Dox's medium (DICKMAN and PATIL 1986) containing 20 % (v/v) of acetone used as potential enzyme extractor (LAURENT 1982). After 2 h, the suspension was filtered again to discard the mycelium.

For esterase activity measurements, *A. pisi* cultures were incubated for 24 to 72 h. At the end of incubation period, distilled water, or acetone used as enzyme extractor, were added (20 % v/v). Incubation was continued for 2 h, and cultures were finally filtered to discard the mycelium.

Cutinase induction

To induce cutinase, the mineral solution of the modified Czapeck-Dox's medium (DICKMAN and PATIL 1986) was neutralized to pH 7 with phosphoric acid, and was supplemented with 0.2 % (w/v) cutin and/or different concentrations of the following commercially available C_{16} and C_{18} fatty acids:

- palmitic acid (hexadecanoic acid) from Sigma,
- juniperic acid (16-hydroxyhexadecanoic acid) from Aldrich-Chemie,
- stearic acid (octadecanoic acid) from Sigma,

— 12-hydroxystearic acid (DL-12-hydroxyoctadecanoic acid) from Aldrich-Chemie,
— ricinelaidic acid ([+]-12-hydroxy-trans-9-octadecenoic acid) from Sigma,
— ricinoleic acid ([R]-12-hydroxy-cis-9-octadecenoic acid) from Sigma.

Cutin was purified from peelings of apples cv. "Golden Delicious", as described by BAKER and BATEMAN (1978). The mineral solution was used as cutinless control medium. All media were autoclaved for 20 min at 120 °C.

Cutinase activity

Cutinase activity released by *A. pisi* was measured by microtitration of the fatty acids released from a cutin substrate. This method was used with *A. pisi* grown on medium containing juniperic acid as sole source of carbon. *A. pisi* culture filtrates were dialysed for 48 h against distilled water, and lyophilized. Lyophilized filtrates were incubated for 24 h at 35 °C in the cutin reaction medium of BASHAN *et al.* (1985), made of 2 ml of 1 mM phosphate buffer pH 7, 1 ml of cutin suspension at 10 mg/ml, and a drop of toluene. The reaction medium was supplemented or not with 10 mM diisopropyl fluorophosphate (DFP), a cutinase activity inhibitor (PURDY and KOLATTUKUDY 1975), and was incubated for 24 h. It was then filtered, and pH of the filtrate was adjusted to 3.5 with phosphoric acid.

Fatty acids in the filtrate (3 ml) were extracted 3 times with diethyl ether, followed by evaporation under vacuum. They were then dissolved in methanol and titrated with NaOH (from the initial pH up to pH 8.2). Palmitic acid was used as standard, and cutinase activity was expressed as units (U)/ml of filtrate (1 U = 1 μg of palmitic acid equivalent/h).

Esterase activity

Cutinase is also evaluated by measuring esterase activity, using *para*-nitrophenyl esters as model substrate (KOLATTUKUDY 1985). In the present work, we evaluated esterase activity using *para*-nitrophenylbutyrate (PNPB) as substrate and *para*-nitrophenol (PNP) as final product, as revealed by measuring absorbance at 400 nm. The enzymatic reaction was carried out in 7 ml of 30 mM phosphate buffer pH 7, mixed with 1 ml of *A. pisi* culture filtrate and 2 ml of 1 mM PNPB in the phosphate buffer (PARKKINEN *et al.* 1978). Esterase activity was expressed as units (U)/ml of filtrate (1 U = 1 μM PNP/min).

Results

Induction of esterase activity with fatty acids

Among the fatty acids used at 0.05 % or 0.5 % as sole source of carbon, juniperic acid proved to be the only powerful inducer of esterase activity release

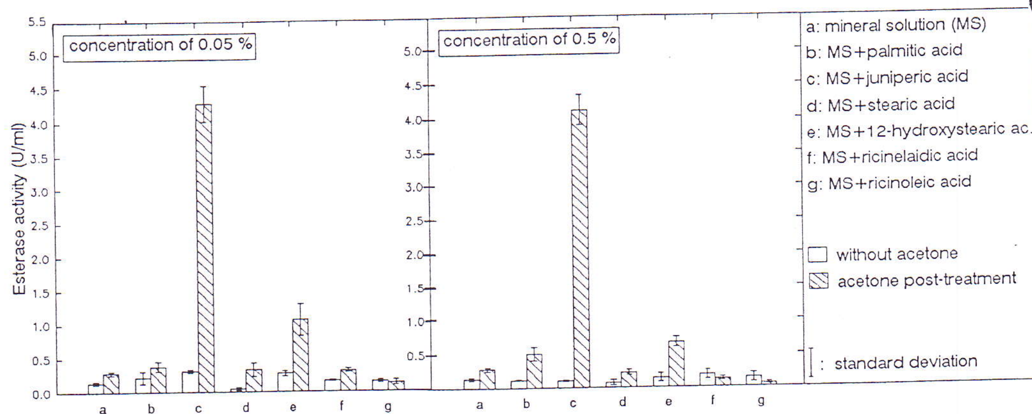

Fig. 1. Esterase activity released by *A. pisi* after 24 h of culture in media containing 0.05 % or 0.5 % of different fatty acids used as sole carbon source, followed or not by acetone post-treatment

after 24 h of incubation, when *A. pisi* cultures were post-treated with 20 % acetone (Fig. 1). Without acetone post-treatment, esterase activity released after 24 h by *A. pisi*, was almost nil for all fatty acids used.

Induction of esterase activity by juniperic acid

When increasing concentrations of juniperic acid were used as sole carbon source, high esterase activity was released after 24 h by *A. pisi*, when cultures were post-treated with acetone (Fig. 2). No significant esterase activity was released without acetone post-treatment.

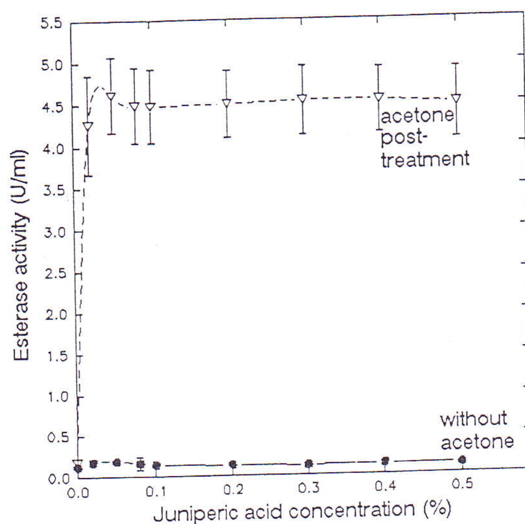

Fig. 2. Esterase activity released by *A. pisi* after 24 h of culture in media containing increasing concentrations of juniperic acids used as sole carbon source, followed or not by acetone post-treatment, (I: standard deviation)

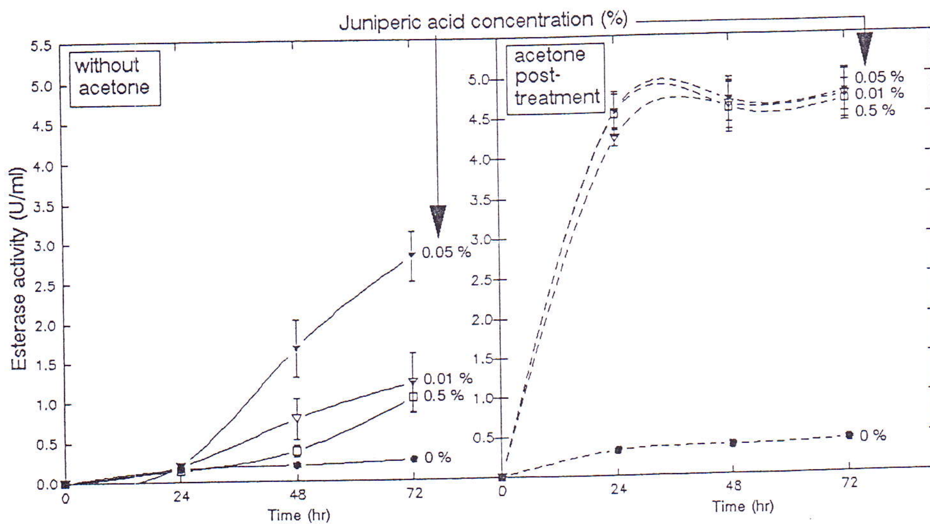

Fig. 3. Esterase activity released by *A. pisi* after culture in media containing different concentrations of juniperic acid used as sole carbon source, followed or not by acetone post-treatment, (I: standard deviation)

When the culture period in juniperic acid medium was extended up to 72 h, the release of esterase activity increased, and was higher with 0.01 % or 0.05 % than with 0.5 % juniperic acid. Without juniperic acid (control), no esterase activity was released (Fig. 3). Acetone post-treatment resulted in the similar release of esterase activity for the three concentrations of juniperic acid.

Cutinase activity induced by juniperic acid

The enzyme released by *A. pisi* grown in culture medium containing juniperic acid (with or without acetone extraction from the mycelium) showed cutinolytic (cutinase) activity, and was inhibited by adding DFP, a cutinase inhibitor, to the enzymatic reaction medium (Table 1).

Table 1
Cutinase activity of *A. pisi,* as measured by the method of microtitration of fatty acids released from cutin substrate[1])

Treatment	Cutinase activity (U/ml)
Control without lyophilized culture filtrate	0.15
Lyophilized culture filtrate	0.60
Lyophilized culture filtrate + DFP	0.18
Lyophilized acetone-treated mycelium filtrate	0.84
Lyophilized acetone-treated mycelium filtrate + DFP	0.15

[1]) *A. pisi* cultures grown for 15 days on juniperic acid medium, were filtered and the filtrate was collected. The mycelium was resuspended for 2 h in fresh mineral solution containing 20 % acetone, and then filtered again. Both filtrates were dialysed, lyophilized and mixed with a cutin suspension for 24 h, in the presence or not of 10 mM of the cutinase inhibitor DFP. The released fatty acids were extracted in diethyl ether, dissolved in methanol, and microtitrated with NaOH.

Inhibition of esterase activity by fatty acids

The addition of 0.05 % fatty acids to cutin in the culture medium did not modify the low esterase activity released by *A. pisi* after 24 h. Post-treatment with acetone induced a large increase of the release of esterase activity by *A. pisi* grown on cutin alone (control), while addition of stearic acid to the cutin medium reduced the release of esterase activity by half. Addition of 0.05 % of other fatty acids to cutin had little effect (Fig. 4).

Addition of 0.5 % fatty acids to the cutin culture medium resulted in low esterase activity in all cases without acetone post-treatment. Post-treatment with acetone largely increased esterase release and reached almost the same level for cutin alone, or for cutin supplemented with palmitic, juniperic or 12-hydroxy-stearic acids. Incubation of *A. pisi* in cutin medium supplemented with 0.5 % stearic acid, released only 1/3 of the esterase activity released in the cutin alone control. When 0.5 % ricinelaidic acid or ricinoleic acid was added to the cutin medium, release of esterase activity dropped to almost nil.

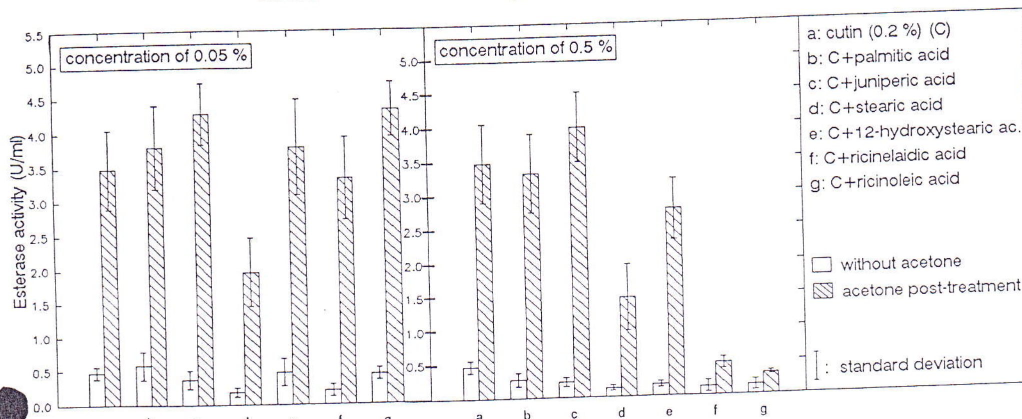

Fig. 4. Esterase activity released by *A. pisi* after 24 h of culture in media containing cutin (0.2 %) supplemented with 0.05 % or 0.5 % of different fatty acids, followed or not by acetone post-treatment

Inhibition of esterase activity by ricinoleic acid

After 24 h of culture, little esterase activity was released by *A. pisi* grown in cutin medium supplemented with increasing concentrations of ricinoleic acid (Fig. 5). Upon post-treatment with acetone, the release of esterase activity which was high at low concentrations of ricinoleic acid in the cutin medium (up to 0.1 %), decreased sharply with increasing ricinoleic acid concentration, to reach almost nil with 0.4 % and 0.5 %.

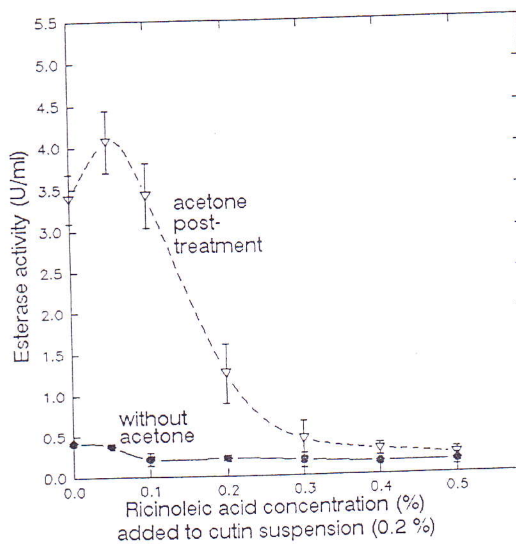

Fig. 5. Esterase activity released by *A. pisi* after 24 h of culture in media containing cutin (0.2 %) supplemented with increasing concentrations of ricinoleic acid, followed or not by acetone post-treatment, (I: standard deviation)

When the culture period was extended up to 72 h, similar results were obtained (Fig. 6).

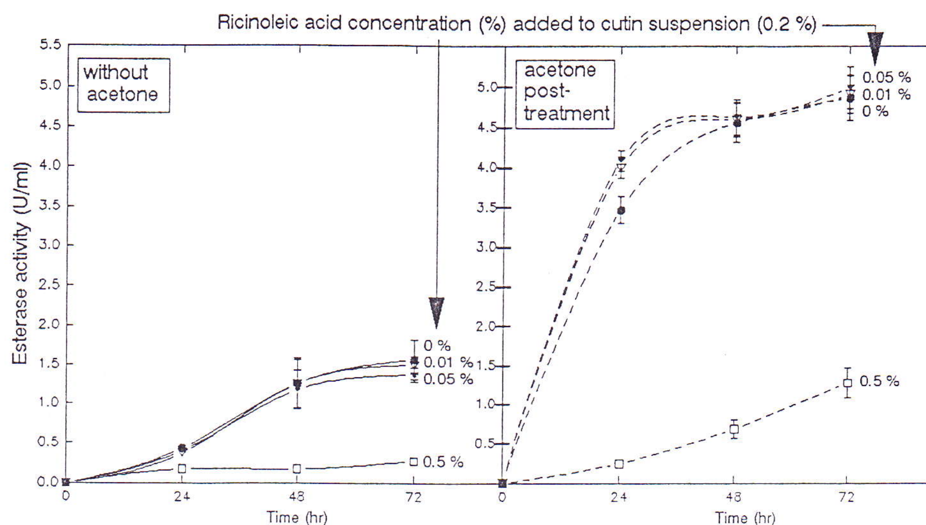

Fig. 6. Esterase activity released by *A. pisi* after culture in media containing cutin (0.2 %) supplemented with different concentrations of ricinoleic acid, followed or not by acetone post-treatment, (I: standard deviation)

Juniperic and ricinoleic acids as carbon source for *A. pisi*

Culture of *A. pisi* for 72 h in a medium containing 0.05 % juniperic acid as carbon source, released an esterase activity which was enhanced by post-treatment with acetone. In contrast, when 0.5 % ricinoleic acid was used as sole carbon source, *A. pisi* did not release a significant esterase activity. When supplemented to medium containing 0.05 % juniperic acid, ricinoleic acid at 0.5 % inhibited completely (without acetone) or strongly (with acetone post-treatment) the release of esterase activity (Fig. 7).

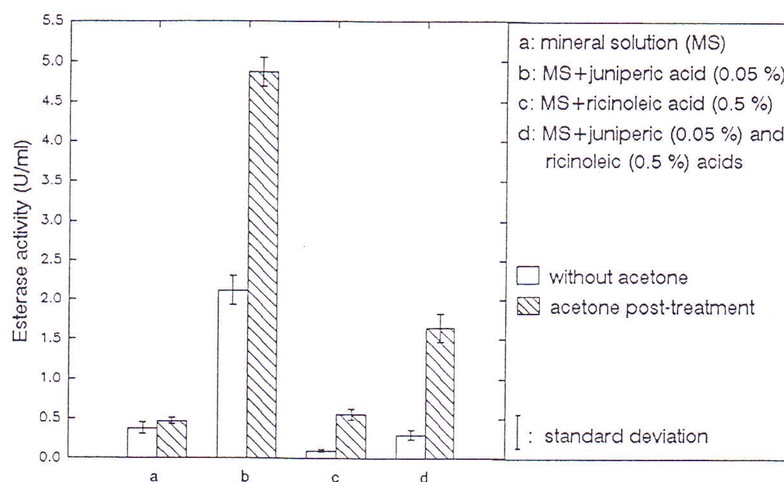

Fig. 7. Esterase activity released by *A. pisi* after 72 h of culture in mineral medium containing 0.05 % juniperic acid and/or 0.5 % ricinoleic acid, followed or not by acetone post-treatment

Discussion

Cutinase has been usually induced by cultivating fungi on cutin as sole carbon source (KOLATTUKUDY 1985, KÖLLER 1991). In the case of *F. solani* f. sp. *pisi*, cutinase was also induced by cutin fatty acids (LIN and KOLATTUKUDY 1978, WOLOSHUK and KOLATTUKUDY 1986, PODILA *et al.* 1988). In the present work, we used commercial fatty acids as carbon source for *A. pisi*, and showed that juniperic acid is a good inducer of the *in vitro* release of esterase activity by this fungus.

Using the method of microtitration of fatty acids released from cutin substrate, we confirmed that the enzymatic activity induced by juniperic acid (with or without acetone post-treatment) is indeed cutinase.

Little cutinase was released by *A. pisi* after 24 h of culture in a mineral medium supplemented with juniperic acid. Cutinase activity increased when the culture period was extended to 72 h, but less cutinase was released in the medium containing 0.5 % juniperic acid, than with 0.01 % or 0.05 %. After acetone post-treatment, the level of released cutinase was the same with 0.01 %, 0.05 % or 0.5 % juniperic acid. These results suggest that juniperic acid induced the synthesis of cutinase at all three concentrations, while the release of this enzyme was partially inhibited by 0.5 % juniperic acid, and restored by post-treatment with acetone.

To study the possible inhibition of cutinase release, the mineral culture medium was supplemented with a mixture of cutin and commercial fatty acids. Ricinoleic and ricinelaidic acids at 0.5 % strongly inhibited cutinase release, even after acetone post-treatment, but were without effect at 0.05 %.

Among all fatty acids tested, ricinoleic acid was the strongest inhibitor of cutinase release by *A. pisi*. At 0.5 %, it severely inhibited the release of cutinase induced by cutin or juniperic acid used as source of carbon, but was without effect at 0.01 % or 0.05 %. This indicates that, in contrast to juniperic acid, ricinoleic acid might inhibit not only the release, but also the induction of the enzyme.

Our overall results suggest that some commercially available C_{16} and C_{18} fatty acids are able to induce or inhibit the *in vitro* release of cutinase by *A. pisi*, and so could play a role in the regulation of the fungal cutinase production during the process of infection of the host-plant.

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