INDUCTION OF BETTER GROWTH AND SPORULATION OF *UROMYCES FABAE* (Pers.) DE Bary GROWN IN AXENIC CULTURE

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ABSTRACT

Modified MS-medium supplemented with broad bean leaf extracts was found to be excellent for growth and sporulation of the two isolates A and B of *Uromyces fabae.*

U. fabae isolate A could utilize a large number of carbon and nitrogen sources than isolate B. Whereas, sucrose and starch, in general, were the best carbon sources for growth and sporulation of both isolates. The bease favorable temperature regimes for growth of *U. fabae* in modified MS medium supplemented with broad bean leaf extracts ranged between 15-27C and between 20-27C for isolates A and B, respectively, with optimal growth at 25C. The effect of relative humidity and pH values at constant temperature (25C) on growth and sporulation as of *U. fabae* isolates A and B were alsostudied. The red, blue and hyaline lights gave the highest significant increase in linear growth and uredospores production of isolate A. However, formation of teleutospores was enhanced under green light wavelengths.

INTRODUCTION

Axenic culture has defined by **Dougherty (1953)** as referring specifically to the growth of one organism free of all others. **Yarwood, (1956**) stated that plant pathologists, plant physiologists, and others have tried unsuccessfully of about 100 years to culture the viruses downy mildew, powdery mildew and rusts on nonliving media. Many regard the task as impossible, and for this reason the term obligate parasites is used to denote these and other organisms which live in such necessary association with living hosts. He defined obligate parasites as organisms, which could not be cultured axenically. He showed that the "hit and miss" methods of finding the correct nutrients for saprophytic cultures to develop from spores had not yet been successful. He suggested that obligate parasites could eventually be cultured, but special techniques will be necessary.

Mains (1917) attempted to grow the rust fungus *Puccenia sorghi* saprophytically by parallel studies of uredospore infection of the host and uredospore development *in vitro*. He postulated that obligate parasites obtain transitory compounds from living hosts, which are necessary for their growth. **Turel & Ledingham (1957)** obtained dense, felt-like growth of aerial mycelium of *Melampsora lini* (Pers.) Lav. when surface-sterilized, rust-infected cotyledons of flax were placed to a modified knop medium, containing calcium nitrate tetrahydrate (500 mg./liter) and ammonium nitrate (10 mg./liter). They added that the effects of the two nitrogen salts were cumulative. **Williams *et al.*, (1967**) noted

that the addition of 0.1% Evan`s peptone to the medium containing yeast extract enhanced vegetative growth from uredospores of wheat stem rust (*Puccinia graminis f.sp. tritici*). Uredospores and teleutospores were formed on stromata. **Bushnell & Stewart (1971**) seeded uredospores of 25 American isolates of *P. graminis tritici* on agar containing peptone, glucose and mineral salts, and incubated these cultures under cool, moist conditions. They found that twenty isolates only formed white mycelial mats indicating development beyond the germ tube stage. **Jones (1972**) reported that extended periods of incubation were necessary for the development of colonies of carnation rust, *Uromyces dianthi*. Axenic cultures of this fungus were grown from uredospores by using media contained yeast extract, peptone and casein hydrolysate, singly or in combination.

Hyphal segments were typically binucleate and growth was optimal at 18-20°C on media of pH 5.8-6.2. He added also, that spore-like cells were formed in the mycelium of older colonies and these spores were capable of germination and of initiation new saprophytic growth. **Scott (1976)** reported that the growth of uredospores sometimes improved by using gelatin suspensions, the addition of bovine serum albumen (BSA) or pectin to the medium. He added that spores rather than vegetative mycelium are the starting material in attempts to culture the rust fungi. **Abu El-Naga (1986**) stated that some physiological races and isolates of *Puccinia graminis tritici*, *P. recondita*, and *P. striiformis* could be grown axenically. The tested races and isolates showed some variation in their growth rate, growth components, margin shape, colony color, medium pigmentation, and cultural exudates. He added that typical uredospores-like cells, and pigmented hypertrophoid mycelia were formed on the different media.

Although the culture of an obligate parasite is an interesting problem in itself, it achieves very little in our ultimate goal of understanding mechanisms of resistance and control of plant diseases unless the cultures are used in appropriate biochemical, nutritional and genetic studies. The present work was conducted to investigate to what the extent, the biotrophic parasitic fungus ***Uromyces fabae,*** the causal of broad bean rust, could be able to grow and sporulate under ***in vitro*** conditions. Some physiological factors affecting growth and sporulation of this pathogen in axenic culture were also investigated.

MATERIAL & METHODS

Two isolates of *Uromyces* sp. namely isolate A and B were isolated from rusted leaves of broad bean (*Vicia faba* L.) plants grown at Moshtohor (Qalubia district) and Abo-Hommos (Beheira district), respectively using the previous described technique (**El-Fiki, et al.**, 1998).

Tested medium:

The MS-medium (**Murashige** and **Skoog**, **1962)** modified by **El-Fiki, et al.** (**1998)** was used in the following studies. The modified MS-medium (Table, 1) was prepared, autoclaved and used for testing growth and sporulation of *Uromyces* sp. in most of the following experiments.

Effect of removing some constituents of the original MS-medium:

In this experiment, the stock solutions D; E; 'E~'; F; G; Hand I were individually omitted from the tested medium (see Table, 1). The different prepared agar MS-media were autoclaved as usual and used to investigate effect of exclusion of these components on growth and uredospores production of *Uromyces* sp. in axenic culture. Both linear growth and uredospores production were determined after 12 days from incubation at 25C. Uredospores produced by a given treatment were harvested and counted by a haemacytometer then, number of uredospores/ml was calculated.

Table (1): Chemical ingredient of the salts of the modified MS-medium by **El-Fiki *et al.* (1998)**

Stock solution	Constituents	Final conc. in medium (mg/L.)
A	$NH_4 NO_3$	825.000
B	KNO_3	685.000
C	$H_3 BO_3$	6.200
	$KH_2 PO_4$	170.000
	KI	0.830
	$Na_2 MoO_4.2H_2O$	0.250
	$CoCl_2.6H_2O$	0.025
D	$CaCl_2.2H_2O$	440.000
E	$MgSO_4.7H_2O$	370.000
	$MnSO_4.4H_2O$	22.300
E”	$ZnSO_4.7H_2O$	8.600
	$CuSO_4.5H_2O$	0.025
F	Na.EDTA	37.250
	$FeSO_4.7H_2O$	27.850
G	Thiamin-HCl	0.100
	Nicotinic acid	0.500
	Pyridoxin-HCl	0.500
	Glycine	2.000
H	NAA	1.000
I	Inositol	100.000
J	Sucrose	30.0g/l

Effect of leaf extract and age of culture:

Boiled extracts of broad bean leaves (200 g leaves per liter of distilled water) were used instead of water for preparing the above-mentioned modified MS-7 medium. The MS-7 medium with or without leaf extracts for determination

of linear growth, mycelial dry weight and uredospores production of isolates A and B after 10, 15 and 21 days from incubation at 25 °C.

Effect of different carbon sources:

Both isolates A and B of *U. fabae* were grown at 25°C on modified MS-medium contained different carbon sources i.e. sucrose, xylose, arabinose, glucose, galactose, fructose, maltose, lactose, starch, mannitol or citric acid (30 g or ml/l). Effects of these carbon sources on linear growth and sporulation of both isolates in axenic cultures were determined after 10 days from incubation at 25°C as mentioned before.

Effect of different nitrogen sources:

Effect of different N sources i.e. $NaNO_3$; KNO_3; NH_4NO_3; $NaNO_2$; $(NH_4)_2SO_4$; urea; asparagine; gelatin (8.889% N); yeast extract (11.0% N); beef extract (12.5% N); casein (13.754% N); and peptone (16% N) were used singly as sole sources of nitrogen. The different N sources were added to the modified MS medium contained at amounts equivalent to the total N (1.238 g N/l) in the modified MS-medium (contained 0.825 g/l of NH_4NO_3 and 6.85 g/l of KNO_3 equivalent to1.238 g N/l) at amounts equivalent to the total N (1.238 g N/l) in this medium. Effect of these N sources on linear growth and sporulation of *U. fabae* was estimated after 10 days from incubation at 25C as mentioned before.

Effect of different temperatures:

Linear growth and uredospore production of *U. fabae* isolates A and B gown on the modified MS-agar medium at different temperature degrees i.e. 5, 10, 15, 17, 20, 23, 25, 27, 30 and 32 °C were investigated. Four replicates were used for each isolate in each temperature.

Effect of relative humidity:

Uromyces fabae isolates A and B were allowed to grow on plates of modified MS-agar medium subjected to different degrees of relative humidity (RH), i.e. 14-100% prepared according to **Solomon** (1951). Aliquots of the specific solution replicated four times were poured into plate lid of the inverted Petri-dishes. All treatments were incubated at 25°C and daily observed. The linear growth and uredospores production were measured after 10 days as mentioned before.

Effect of pH:

The used modified MS-agar medium was prepared in acetate or phosphate buffer at different pH values prepared as described by **Ju-Luric** (1978) and autoclaved in the usual way. The pH of the media was checked after sterilization. The media were poured in plates, inoculated with an equal discs (6mm in diam.) of the tested isolate A and B and incubated 25°C for 7-9 days. Three replicates were made for each treatment. Linear growth and uredospores production were estimated as mentioned before.

Effect of light:

Petri-dishes with modified MS-agar medium were inoculated *U. fabae* isolates A and B, enveloped with colored thin transparent cellophane paper i.e. hyaline, yellow, red, green, blue or in thick black paper then divided into two groups. The first group was incubated at 25 °C under continuously illuminated 60 cm long white fluorescent lamp, hanged 30cm high above the plates inside the incubator Four replicates were used for each particular treatment. In both groups the linear growth and uredospores production were determined as mentioned before.

Statistical analysis:

Statistical analysis was determined as described by **Kohler *et al.*, (1984)**..

RESULTS AND DISCUSSION

Effect of removing some constituents of MS-medium:

Data in **Table** (**1**) proved that both isolates A and B of *U. fabae* were significantly varied. Isolate A produced the highest values of growth rate and number of uredospores. Removing solution D (contained $CaCl_2.2H_2O$) from MS-medium improved linear growth and uredospores production in both *U. fabae* isolates A and B compared with the complete (control) medium (Fig.1). On the other hand, removing of the solution E" (contained $MnSO_4.4H_2O$, $ZnSO_4.7H_2O$, and $CuSO_4.5H_2O$) resulted in the highest reduction in both linear growth and uredospores production especially in isolate A while removing solutions E (contained $MgSO_4.7H_20$), F (contained Na.EDTA and $FeSO_4.7H_2O$), G (contained Thiamin-HCl; Nicotinic acid; Pyridoxin-HCl and Glycine), H (contained NAA) or I (contained myo-inositol) showed intermediate effects in this respect. It was interested to state that the uredospores production was very tiny and was not affected by removing of any stock solution if compared with the normal MS- medium in case of isolate B. Results about effect of calcium conflicted with **Coffey and Allen (1973)** obtained axenic culture of *Puccinia helianthi* from uredospores on artificial media. This fungus had an obligatory requirement for bovine serum albumin (BSA)) and enhanced in the presence of calcium ions. The growth of this organism is characterized by a long lag phase, but once growth had begun it continued for periods up to 25 weeks with individual colonies attaining sizes up to 12 mm. At this time cultures died off unless subcultured. The present findings could be explained in light of the phenomenon of ion-antagonism. Calcium ions might be retarded assimilation of other necessary metallic ions. **Bilgrani** and **Verma (**1981**)** cited that more careful investigations pertaining to the role of calcium in fungal nutrition in general is very much called for. This is particularly so because calcium is often regarded to influence various organisms including fungi, through its non-nutritional role in ion-antagonism against certain toxic monovalent cations. Adverse effect due to removing $MnSO_4$, $ZnSO_4$, and $CuSO_4$ from the growth medium could be attributed the importance of Mn, Zn, and Cu ions in metabolic path way of the tested pathogen.

Table (1): Effect of removing some constituents from the modified MS-medium (control) on linear growth (in mm) and sporulation of *Uromyces fabae* isolates A and B

Removed constituent	Linear growth "mm" **		Number of uredospores/ml	
	Isolate A	Isolate B	Isolate A	Isolate B
Control	60.0	10.0	15448	138
- D	70.0	20.0	17694	628
- E	63.0	8.0	7685	190
- E"	47.0	8.0	6069	288
- G	53.0	8.0	10331	163
- H	59.0	8.0	11609	135
- Inositol	56.0	7.0	5390	238
LSD. at 0.05	1.5		1515	

** Produced from 6 mm diameter original inoculum disc.

Effect of leaf extract and age of culture:

The obtained results **(Table 3)** show clearly that the linear growth of isolate B was greatly enhanced on modified MS-medium provided with broad bean leaf extracts compared with the control medium (without broad bean leaf extracts). As for isolate A the same trend was observed with 10 days-old culture only, while no variations were detected in both 15 and 21 days-old cultures. The growth of isolate A on slops of modified MS-medium was darker than isolate B. The mycelial dry weight of both isolates A and B was increased with aging and greatly stimulated by broad bean extracts added to the modified MS-medium. Uredospores production was similarly affected, however, the highest numbers of uredospores was produced by 21 days-old cultures of both isolates particularly in modified MS-medium containing broad bean leaf extract.

It could be concluded that, adding broad bean leaf extracts to the modified MS-medium enhanced linear growth, dry weight of mycelia and production of uredospores especially in isolate B. The latter isolate may needs special unknown nutritional requirements which found in broad bean leaf extracts but not in the tested modified MS- medium. **Ray** (1901 & 1903) obtained the axenic growth of rusts on agar-solidified media containing decoctions of host tissues or carrots. **Ezekiel** (1930) stated also that host extracts were nutritive to *Puccinia graminis* only when very dilute. Host decoctions has to be diluted before germination was possible, and greatly diluted before a statistical increase in germ tube elongation was observed. It is obvious that ordinary methods of extracting plant sap are very and probably yield many substances, which the invading parasite does not encounter in natural infection.

Table (3**):** Effect of modified MS-medium alone (control) or provided with broad bean leaf extract on linear growth, mycelial dry weight and number of uredospores of *Uromyces fabae* isolates A an B at different ages of cultures.

Age	Linear growth(mm) **		Dry weight of growth (mg)		No. Of uredospores/ml	
	Isolate A	Isolate B	Isolate A	Isolate B	Isolate A	Isolate B
On modified MS-medium (control)						
10 days	61.0	6.5	0.177	0.080	6063	127
15 days	70.0	8.5	0.247	0.103	9946	329
21 days	70.0	22.0	0.270	0.153	12842	457
LSD at 0.05	4.5		0.06		1818	
On modified MS-medium with broad bean leaf extract						
10 days	67.0	37.0	0.230	0.153	10923	3721
15 days	70.0	61.8	0.247	0.163	12142	5790
21 days	70.0	70.0	0.400	0.173	14292	8696
LSD at 0.05	2.9		0.22		3721	

** Produced from 6 mm diameter original inoculum disc.

Effect of different carbon sources:

Data in **Table (4)** stated that both linear growth and uredospores formation were significantly affected by kind of the tested carbon sources. All tested carbon sources except acetic acid were utilized by *U.fabae* isolate A meanwhile starch was the superior followed by sucrose only in case of isolate B. In this regard

the best linear growth and uredospores production were obtained when sucrose and starch were used as sole sources of carbon for *U. fabae* isolae A and B, respectively.

Table (4): Effect of different carbon sources on linear growth and number of uredospores produced by the two isolates A & B of *Uromyces fabae*.

	Linear growth "mm"		Number of uredospores/ml	
Carbon sources	Isolate A	Isolate B	Isolate A	Isolate B
Without 0-carbon	6	6	0	0
Xylose	47	6	5940	0
Arabinose	39	6	4535	0
Glucose	46	6	5679	0
Galactose	53	6	6496	0
Fructose	41	6	5625	0
Maltose	46	6	3700	0
Lactose	47	6	7692	0
Sucrose	70	7	12515	88
Starch	56	40	2975	231
Mannitol	46	6	4567	0
Citric acid	0	0	0	0
L.S.D. at 05	0.9		764	

** Produced from 6 mm diameter original inoculum disc.

Effect of different nitrogen sources:

Data in **Table (5)** showed that the isolates A and B of *U. fabae* were responded differently against tested nitrogen sources. The isolate A could utilize most tested nitrogen sources when added to modified MS-medium while isolate B could utilize asparagine, yeast and beef extracts only. The highest linear growth and uredospores production of isolate A was obtained in control modified MS-medium (contained potassium nitrate + ammonium nitrate) followed by media contained either beef extract, yeast extract, peptone, or asparagine as sole source of nitrogen. However, the highest linear growth of isolate B was produced on modified MS-media containing beef extract, yeast extract, or asparagine but yeat extract was the best for its uredospores production of isolate B.

Table (5): Effect of different tested nitrogen sources on linear growth and number of uredospores produced by the two isolates A & B of *Uromyces fabae*.

	Linear growth (mm) **		No. of uredospores / ml	
Nitrogen Source	Isolate A	Isolate B	Isolate A	Isolate B
Control (MS-7)	70	7	12505	88
Sodium nitrate	44	6	4206	0
Potassium nitrate	65	6	8617	0
Ammonium nitrate	44	6	5160	0
Sodium nitrite	6	6	0	0
Ammonium sulfate	10	6	138	0
Urea	44	7	5180	0
Aspargine	65	31	8479	2188
Gelatin	42	7	3592	0
Yeast extract	70	32	10106	4744
Beef extract	70	60	11025	1098
Casin	59	7	2550	0
Peptone	70	6	8356	0
LSD.05	1.2		1285	

** Produced from 6 mm diameter original inoculum disc.

On the other hand, sodium nitrite seems to be toxic for both isolates A and B. These results are in agreement with **Turel** and **Ledingham** (1957) who obtained aerial mycelium of *Melampsora lini* (Pers.) Lav. on a modified knop medium, containing Difco Bacto-Agar (0.8-1.0%); turbid whole coconut milk from frish, ripe nuts (10%); calcium nitrate tetrahydrate (500 mg./liter); ammonium nitrate (10 mg./liter); adenine (10 mg./liter); and sucrose (4%). They added that the effect of of the two nitrogen salts were cumulative as were those of optimal sugar content and high grade coconut milk.. In fact, difference in the value of various types of nitrates is obviously due to different cations involved in these compounds (**Bilgraniand** and **Verma**, 1981). The present results indicated also that KNO_3 was better for growth and sporulation of isolate A than NH_4NO_3.

Effect of temperature:

Table (6): Effect of temperature on linear growth and number of uredospores produced by the two isolates A & B of *U. fabae* grown on modified MS-medium with or without broad bean leaf extract.

	Modified MS-medium				Modified MS-with leaf host extracts			
	Growth "mm"		Spores/ml		Growth "mm"		Spores/ml	
C. °	Isolate A	Isolate B	Isolate A	Isolate B	Isolate A	Isolate B	Isolate A	Isolate B
5	12	6	134	106	17	9	184	91
10	13	9	153	178	20	14	434	191
15	25	17	1084	206	33	19	2616	1606
17	27	17	4475	225	35	21	10897	7491
20	37	17	9941	672	47	54	12259	13369
23	52	9	15303	397	63	58	16356	18472
25	61	7	13269	189	67	66	20803	14469
27	57	7	4231	79	59	50	12134	1519
30	32	7	3900	72	25	28	4206	1178
32	24	6	3122	63	14	13	3791	1081
L.S.D.	3.8		2267		5.3		3027	

** Produced from 6 mm diameter original inoculum disc.

Data in **Table (6)** show that isolates A and B of *U. fabae* produced the highest linear growth at 25 °C meanwhile the highest production of uredospores was obtained at 25 °C for isolate A and 23 °C for isolate B when they were grown on modified MS-medium provided with broad bean leaf extract. This trend was slightly differed when these isolates grown on modified MS-medium only (with broad bean leaf extract). On the latter medium It could be observed in general that, the best linear growth of isolate A was obtained at 25 C follwed by 27 and 23 °C, while temperatures of 15, 17 and 20 °C gave the best results in case of isolate B. The uredospores production of the later isolates was not significantly affected by different regime temperatures when grown on the present modified MS-medium alone (without broad bean leaf extracts).

These results could be understood in light of knowledge of the average of temperature in different districts of Egypt. In Qlubia district from which isolate A was isolated, the average temperature was, in general, higher during February and March when the broad bean rust disease appear than that in Beheira district, from which isolate B was isolated.

Effect of relative humidity:

The data in **Table (7)** proved that all tested degrees of constant relative humidity conditions ranged between 14-100% R.H. caused significant decreases in both linear growth and production of uredospores of isolates A and B of *U. fabae*. Further significant reduction in uredospores production by isolate A was noticed by increasing relative humidity from 95 to 100% R.H. In case of isolate B only, which grew slower than isolate A, the highest value of linear growth was obtained in control treatment followed by treatments of 74 and 50% R.H., respectively without significant differences. It could be concluded that fluctuation in relative humidity may be necessary for growth and sporulation of *U fabae* in axenic culture. Both linear growth and uredospore formation of both isolate A and B were sharply and significantly decreased when they were grown under constant controlled relative humidity conditions (14-100% R.H.).

Table (7): Effect of relative humidity on linear growth and number of uredospores produced by the two isolates A & B of *Uromyces fabae*.

	Linear growth		Number of uredospores /ml	
R.H %	Isolate A	Isolate B	Isolate A	Isolate B
Control *	60.0	11.0	15448	138
14 %	27.7	6.0	1013	19
50 %	33.8	9.7	1752	25
74 %	33.2	10.4	1313	13
80 %	24. 3	7.5	1273	13
85 %	24. 3	7.5	1238	10
90 %	24.0	7.3	1237	8
95 %	24.0	7.0	1092	8
100 %	22.5	6.2	354	0
L.S.D. at 0.05	1.5		570	

Control * = Uncontrolled R.H. conditions. ** Produced from 6 mm diameter original inoculum disc.

8-Effect of PH values:

The data in **Table (8)** illustrated clearly that the pH values which favored growth and uredospores production on modified MS-medium were lying between 6.6-9.0 in case of *U. fabae* isolate A and 8.0-9.9 in case of isolate B. Both isolates had no grown or sporulated at lower pH values i.e. 4.0-4.6. **Jones (1972)** reported that hyphal segments of carnation rust, *Uromyces dianthi* grown in axaxenic cultures from uredospores by using media containing yeast extract, peptone and casein hydrolysate singly or in combination were typically binucleate and growth was optimal at 18-20°C on media of pH 5.8-6.2.

Effect of light wavelength:

Data in **Table (9**) indicated that the hyaline wavelength was best for linear growth followed by red, blue, yellow and green wavelengths, respectively. Meanwhile, darkness (black) produced the lowest values of linear growth. As for uredospores production, the red followed by blue and hyaline wavelengths produced the highest numbers of uredospores. No zonation was observed, however, formation

of teletospores was enhanced by green wavelength, (**Fig. 1**). These results are in partial agreement with **Leach (**1962**)** who studied the effect of irradiation on sporulation of different species of fungi, and observed that in 34 fungal species, sporulation was effectively induced by near ultra-violet radiation and was better than that by longer wavelengths, and even long exposures did not prove lethal or inhibitory.

Table (8): Effect of pH values on linear growth and number of uredospores produced by the two isolates A & B of Uromyces fabae on modified MS-medium.

PH values	Linear growth		Number of uredospores	
	Isolate A	Isolate B	Isolate A	Isolate B
4.0	6.0	6.0	0	0
4.6	6.0	6.0	0	0
5.0	39.5	6.0	4823	31
5.6	59.0	7.0	7113	56
6.0	59.5	7.0	7350	56
6.6	66.6	7.0	11098	67
7.0	67.9	7.0	12189	81
7.6	66. 7	7.5	12292	196
8.0	68.1	15.3	13008	710
8.6	68.8	18.4	12780	1108
9.0	69.0	17.8	11856	1081
L. S. D. 0.05	1.8		1892	

** Produced from 6 mm diameter original inoculum disc.

Table (9): Effect of different wavelengths on linear growth and number of uredospores produced by *Uromyces fabae*- isolate A

	Linear growth (mm) **		Number of uredospores / ml	
wavelength	Continues (1)	Day light (2)	Continues (1)	Day light (2)
Hyaline	67	35	12917	3528
Yellow	61	25	9987	1647
Red	64	40	15308	3876
Green	54	22	8100	1614
Blue	64	44	14947	5815
Black	51	32	9631	4438
Mean	60.2	33.0	11815	3486
L.S.D. at 0.05	1.9	1.9	2060	727

** Produced from 6 mm diameter original inoculum disc.

(1) = continues illumination under controlled temperature conditions.

(2) = normal day diffused light (day + night) under room temperature conditions.

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