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Research Article

Evaluation of Pathological Variations in *Alternaria* Species Infecting Oilseed *Brassicas* in Diverse Regions of India for Induction of Systemic Resistance

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Abstract

The 32 isolates of *A. brassicae*, 20 isolates of *A. brassicicola* and 3 isolates of *A. alternata* originating from diverse regions of North-West India, where *Alternaria* blight is highly prevalent, were investigated for their pathogenicity on various host species. The disease reaction varied with necrotic lesions of 0.4-7.5 mm, mostly having brown to black coloured lesions. The isolates caused moderate to severe chlorosis on *B. rapa* varieties (YSH-401 and Pusa gold) as compared to the *B. juncea* varieties (Varuna, Rohini, Kranti and PHR-01). The mean disease index varied from 2.22 ± 1.17 to 7.32 ± 0.67 in *A. brassicae* isolates, 2.38 ± 0.92 to 5.89 ± 0.89 in *A. brassicicola* isolates and 2.49 ± 0.97 to 5.87 ± 0.91 in *A. alternata* isolates. Out of seven *A. brassicae* isolates that tested positive for non-aggressiveness on specific host species, five isolates, on prior inoculation, induced tolerance to highly aggressive strains of *A. brassicae* in the respective *B. juncea* and *B. rapa* varieties. The study thus opens up the possibility of deploying attenuated virulence as naturally occurring biological control for induction of systemic resistance/ tolerance in *Brassica*s against *A. brassicae*, one of the most destructive fungal pathogen.

Keywords: *Alternaria* blight; Oilseed *Brassica*; Pathogenicity; Attenuated virulence; Systemic resistance

Abbreviations

PDA: Potato Dextrose Agar; SA: Salicylic Acid; JA: Jasmonic Acid; ISR: Induced Systemic Resistance; PR: Pathogenesis-Related; DI: Disease Index.

Introduction

Pathogens react vigorously to their environment; even the slightest variations in the environment may greatly impact the pathogen and result in minute to massive adaptations in its population [1]. The extent and rate of such adaptations brought about in a pathogen shapes its morphological and physiological characteristics, and also changes its behavior towards the host population. *Alternaria* species have been extensively studied for their differential pathogenicity/ virulence on different host species. Variability in virulence towards host species have been reported in isolates of *A. brassicae* [2-9] *A. brassicicola* [3,10,11], *A. alternata* [12] and *A. solani* [4,13,14]. The availability of pathotypes with varying degrees of pathogenicity towards different *Brassica* species and sub-species acts as an important aspect in identification, breeding and exploitation of durable resistance genotypes.

Induction of resistance in otherwise susceptible host plants, without changing their basic genetic make-up, through use of biotic as well as abiotic agents has been studied by a few scientists [15,16]. The resistance/ defense related genes in the vulnerable plants can be activated by inoculating the plant either by an avirulent form of the pathogen or by limited inoculation with the pathogen [17]. Infecting avirulent pathogen triggers natural defense responses in the plant through the release of the elicitors which then result in the expression of novel anti-pathogenic proteins.

In order to harness the benefits of induced host resistance and build up a stable, long term resistance mechanism in the host plant against the pathogen, there is a need to identify the pathogen and understand its behavior under diversified conditions. However, only a few studies [7] have been undertaken to evaluate the pathological diversity of the *Alternaria* isolates and its characterization for its applicability in India.

Materials and Methods

Raising *Brassica* plants

Six varieties of oilseed *Brassica* – *B. juncea* (4 varieties - Varuna, Rohini, Kranti and PHR-01) and *B. rapa* (2 varieties – YSH-401 and Pusa gold) were selected for evaluating variations in virulence/ aggressiveness of different isolates. The seeds of the selected varieties were sown in agropeat: sterile soil mix and plants were raised under natural conditions in field during Rabi Season and controlled conditions at 22 ± 2°C with 16 hr light/ 8 hr dark photoperiod and a light intensity of 12klx.

Isolation and purification of fungal cultures

Samples of *Alternaria* blight infected leaves from various *Brassica* species were collected/ procured from Northern and North-western regions of India. The fungal cultures were isolated and purified on PDA (potato dextrose agar) medium under continuous diffused light conditions, at a temperature of 20 ± 2°C for 15 ± 2 days. The purified *Alternaria* isolates were observed microscopically under 40×

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Table 1: Disease assessment scale on the basis of lesion size and intensity of chlorosis.

Disease score	Pathological reaction
0	No lesion formation and absence of chlorosis
1	Lesion size <1mm with slight or no chlorosis
2	Lesion size <1mm with moderate to severe chlorosis
3	Lesion size 1-2mm with slight or no chlorosis
4	Lesion size 1-2mm with moderate to severe chlorosis
5	Lesion size 2-3mm with slight or no chlorosis
6	Lesion size 2-3mm with moderate to severe chlorosis
7	Lesion size 3-4mm with slight or no chlorosis
8	Lesion size 3-4mm with moderate to severe chlorosis
9	Lesion size >4mm with moderate to severe chlorosis

optical microscope and identified at species level as per the available monograph [18].

Pathogenicity assay

The differential virulence/ aggressiveness of the isolates were determined using detached leaf according to the method of Vishwanath and Kolte [19]. The maintained isolates were used to prepare individual spore suspensions (1.5×10^4 spores per ml). Third/ fourth fully expanded leaf from base of 30-day old plants of each variety were detached, inoculated with 10 µl of individual spore suspensions and incubated for 7 days at 22 ± 2°C and 12 hour photoperiod in moist chambers.

The response of different isolates on the selected hosts was assessed on the basis of lesion number, size and colour; presence of ring/ dots in the lesion; chlorotic zone (yellow halo) and latent period of infection, in terms of Disease Index (DI) on a scale of 0-9 (Table 1). The isolates were subsequently characterized as highly aggressive, aggressive or non-aggressive on the host differentials and analyzed statistically for significant variations, if any. On the basis of non-aggressiveness shown by the isolates, three varieties of *Brassica* – *B. juncea* (2 varieties – Rohini and PHR-01), and *B. rapa* (1 variety – YSH-401) were selected for further evaluations.

Evaluation for induction of systemic acquired resistance

Assay for evaluation of systemic resistance induced by attenuated virulence was carried out using detached leaf method. A separate assay was carried out for each isolate showing non-aggressive behavior on a specific *Brassica* variety. The detached leaves from base of 30-day old plant of the respective *Brassica* variety were inoculated with 10 µl of four different suspensions:: sterile distil water as control (C); spore suspensions of non-aggressive isolate; highly aggressive isolate; non-aggressive isolate (on 1st day of inoculation) followed by spore suspension of highly aggressive isolate (on 3rd day of inoculation). The inoculated leaves were incubated for 10 days at 22 ± 2°C and 12 hr light/ 12 hr dark photoperiod in moist chambers. Host response to pathogen's aggressiveness and disease score was assessed as earlier and data was analyzed statistically for induction of systemic resistance.

Figure 1: Pathological variation in *A. brassicae* isolates – (i-viii) ABc-P05, ABc-P06, ABc-P07, ABc-P08, ABc-P09, ABc-P10, ABc-P11 and ABc-P12 respectively, on different host differentials. From left to right - *B. juncea* var. Varuna, Rohini, Kranti and PHR-01; *B. rapa* var. YSH401 and Pusa gold. Left (a-h) and right side (i-p) show the healthy and inoculated leaves, respectively.

Table 2: Mean disease index (0–9 scale) on 4 varieties of *B. juncea* and 2 varieties of *B. rapa*, 7 days after inoculation with 32 isolates of *A. brassicae.*

A. brassicae isolates	*B. juncea* varieties				*B. rapa* varieties		Mean D.I ±S.E (a)
	Varuna	Rohini	Kranti	PHR-01	YSH-401	Pusa gold	
ABc-D01	3.33	3.66	4.66	3.33	3.0	2.66	3.44±0.31
ABc-D02	1.33	1.33	3.33	3.66	3.66	5.66	3.16±0.73
ABc-D06	6.66	5.66	5.33	0.0	3.66	8.33	4.94±1.28
ABc-D07	3.33	5.33	0.66	0.33	4.0	5.66	3.21±1.02
ABc-P01	6.66	0.0	5.33	7.33	3.33	3.66	4.39±1.19
ABc-P04	2.66	2.66	5.66	4.0	2.33	8.0	4.21±0.99
ABc-P05	5.33	3.0	4.66	3.33	9.0	8.66	5.66±1.16
ABc-P06	1.0	4.66	8.0	3.33	9.0	6.0	5.33±1.33
ABc-P07	1.66	3.66	5.66	0.0	3.66	3.66	3.05±0.87
ABc-P08	3.66	7.66	5.66	5.33	8.33	5.66	6.05±0.76
ABc-P09	7.33	7.66	8.33	8.33	5.66	5.66	7.16±0.55
ABc-P10	5.66	8.66	8.66	7.33	5.33	8.33	7.32±0.67
ABc-P11	8.33	6.66	4.66	3.66	3.33	3.66	5.05±0.90
ABc-P12	2.66	6.66	5.33	0.0	7.0	5.33	4.49±1.2
ABc-L01	4.66	8.66	8.66	2.66	5.0	9.0	6.44±1.2
ABc-L02	2.66	2.33	8.33	6.33	2.66	5.0	4.55±1.09
ABc-L05	3.0	6.33	2.33	8.0	5.3	5.66	5.10±0.94
ABc-L07	1.0	0.66	2.66	3.33	3.66	3.66	2.49±0.60
ABc-L08	2.66	3.0	5.66	7.33	5.33	3.66	4.60±0.81
ABc-L10	2.66	2.33	2.66	3.0	2.33	5.33	3.05±0.51
ABc-L12	6.66	8.33	6.66	5.0	5.33	6.0	6.33±0.53
ABc-L19	2.66	2.66	2.0	3.33	5.33	2.66	3.02±0.52
ABc-L20	6.66	4.66	4.66	5.33	5.66	3.0	4.9±0.55
ABc-B01	3.0	3.0	4.33	0.0	3.66	5.33	3.22±0.81
ABc-B03	2.66	2.66	3.66	0.66	3.33	5.66	3.11±0.73
ABc-H03	3.0	0.66	0.33	0.33	5.33	6.0	2.60±1.15
ABc-H04	2.66	6.66	5.33	0.0	5.66	8.33	4.77±1.34
ABc-H05	5.33	2.66	5.33	0.0	0.0	0.0	2.22±1.17
ABc-H06	2.66	2.66	5.0	2.66	8.0	4.0	4.16±0.94
ABc-Kg01	1.33	2.66	2.66	2.33	2.66	3.33	2.49±0.29
ABc-Kg02	2.66	2.66	3.66	3.66	2.33	3.66	3.11±0.28
ABc-Kn01	2.33	3.0	2.33	3.33	5.3	3.0	3.22±0.49
Mean D.I ±S.E (b)	3.68±0.36	4.15±0.44	4.76±0.39	3.35±0.47	4.63±0.37	5.13±0.38	

Mean DI ±S.E (a) is the average of the disease score produced by each isolate on 3 leaves of each of the host differentials and Mean DI ±S.E (b) is the average of the disease score of all the isolate on 3 leaves of specific host differential.

Results

Pathological diversity

A wide variation was exhibited by *A. brassicae*, *A. brassicicola* and *A. alternata* isolates from different regions on the selected host differentials. The disease reaction varied with necrotic lesions of 0.4 - 7.5 mm, mostly having brown to black colour. A few isolates also formed grey to dark grey coloured lesions. The isolates in general caused moderate to severe chlorosis on *B. rapa* varieties (YSH-401 and Pusa gold) as compared to the *B. juncea* varieties (Varuna, Rohini, Kranti and PHR-01), on which the chlorosis was either absent or was very slight.

On a scale of 0-9, disease severity in *A. brassicae* isolates varied between 2.22 ± 1.17 to 7.32 ± 0.67. *A. brassicae* isolates showed maximum mean disease index of 5.13 ± 0.38 on *B. rapa* var. Pusa gold, followed by *B. juncea* var. Kranti, *B. rapa* var. YSH-401, *B. juncea* var. Rohini, Varuna and minimum value of 3.35 ± 0.47 on *B. juncea* var. PHR-01 (Figure 1, Table 2). Similarly, *A. brassicicola* isolates varied in aggressiveness from 2.38 ± 0.92 to 5.89 ± 0.89. *A. brassicicola* isolates showed highest disease index of 5.05 ± 0.51 on *B. juncea* var. Kranti, followed by *B. rapa* var. YSH-401, Pusa gold,

Table 3: Mean disease index (0–9 scale) on 4 varieties of *B. juncea* and 2 varieties of *B. rapa*, 7 days after inoculation with 20 isolates of *A. brassicicola*.

A. brassicicola isolates	*B. juncea* varieties				*B. rapa* varieties		Mean D.I ±S.E(a)
	Varuna	Rohini	Kranti	PHR-01	YSH-401	Pusa gold	
ABo-D03	0.33	0.33	8.66	9.0	2.66	2.66	3.94±1.78
ABo-D04	2.65	1.0	3.66	3.66	3.66	7.66	3.72±0.98
ABo-D05	5.3	5.66	3.0	5.33	4.0	4.0	4.55±0.47
ABo-D08	0.33	2.0	5.33	0	2.66	4.0	2.38±0.92
ABo-D09	3.66	0.66	2.66	2.66	3.33	4.0	2.82±0.53
ABo-D10	3.0	5.33	5.66	5.33	3.0	5.66	4.66±0.58
ABo-P02	5.67	6.0	5.66	3.66	5.66	7.33	5.66±0.53
ABo-P03	6.0	3.33	5.33	6.0	8.33	2.66	5.28±0.92
ABo-L03	5.0	5.33	2.66	7.33	9.0	3.0	5.39±1.20
ABo-L04	5.33	3.0	8.33	8.0	5.33	5.33	5.89±0.89
ABo-L06	5.33	7.33	5.33	2.33	8.0	3.66	5.33±0.96
ABo-L09	3.0	3.0	2.66	3.0	3.0	3.0	2.94±0.06
ABo-L11	5.0	2.66	3.0	3.0	2.66	4.33	3.44±0.44
ABo-L13	4.66	1.0	8.66	5.33	3.0	8.0	5.11±1.30
ABo-L15	3.0	3.0	6.0	3.0	2.66	7.33	4.17±0.89
ABo-L16	2.66	5.33	3.0	5.33	7.0	0	3.89±1.18
ABo-L17	3.0	2.66	4.66	3.33	2.66	2.66	3.16±0.35
ABo-L18	3.0	6.66	3.0	2.33	3.0	3.66	3.61±0.69
ABo-H01	2.33	4.66	4.66	2.66	9.0	3.0	4.39±1.06
ABo-H02	7.33	0.66	9.0	0.66	5.33	2.66	4.27±1.57
Mean D.I ± S.E(b)	3.83±0.42	3.5±0.51	5.05±0.51	4.1±5.34	4.7±0.54	4.23±0.47	

In the above table, Mean DI ±S.E (a) is the average of the disease score produced by each isolate on 3 leaves of each of the host differentials and Mean DI ±S.E (b) is the average of the disease score of all the isolate on 3 leaves of specific host differential.

B. juncea var. PHR-01, Varuna and a minimum value of 3.51 ± 0.51 on *B. juncea* var. Rohini (Table 3). Among the three *A. alternata* isolates, disease severity varied from 2.49 ± 0.97 to 5.87 ± 0.91. *A. alternata* isolates showed maximum disease index of 6.0 ± 2.1 on *B. rapa* var. Pusa gold followed by YSH-401, *B. juncea* var. Kranti, PHR-01 Rohini and a minimum value of 1.87 ± 1.15 on *B. juncea* var. Varuna. Overall, among the three species, isolates ABc-P10 (*A. brassicae* isolated from Pantnagar), ABo-L04 (*A. brassicicola* isolated from Ludhiana) and ABa-L14 (*A. alternata* isolated from Ludhiana) were most aggressive on all the tested host differentials. Based on their aggressiveness the isolates were subsequently categorized into four groups; non-aggressive, less aggressive, moderately aggressive and highly aggressive on specific host differentials (Table 4).

Induction of systemic resistance against *A. brassicae* isolates

Inoculation of spore suspension of non-aggressive *A. brassicae* isolates prior to inoculation with highly aggressive isolates resulted in the significant reduction in disease severity (at $P < 0.05$) by five out of the seven tested non-aggressive isolates in respective *Brassica* varieties (Figure 2). In case of *B. juncea* var. Rohini, inoculation with a non-aggressive isolate (ABc-P01) prior to highly aggressive isolate (ABc-P10), resulted in 44.5 % reduction in disease severity, compared to the inoculation with highly aggressive isolate alone. Similarly, in *B. juncea* var. PHR-01, non-aggressive isolates (ABc-D06, ABc-P07, ABc-B01 and ABc-H05) reduced the disease severity of highly aggressive isolate ABc-P09 by 7.4, 51.9, 3.7 and 11.1 %, respectively. Isolate ABc-H05 also reduced the disease severity against highly aggressive ABc-P06 in *B. rapa* var. YSH-401 by 18.5 %, thereby inducing systemic resistance. However, in contrast to above, two non-aggressive isolates, ABc-P12 and ABc-H04 acted in an additive manner increasing the disease response in *B. juncea* var. PHR-01 by

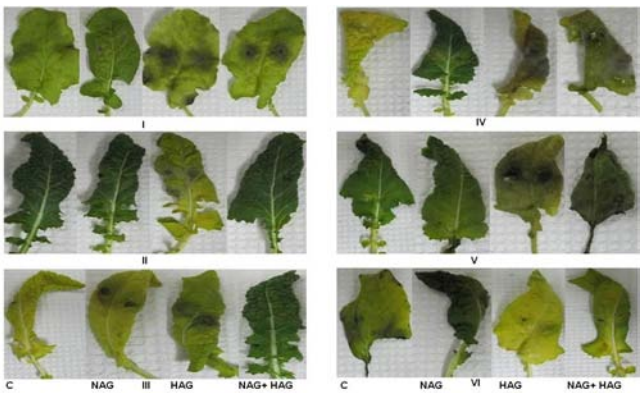

Figure 2: Disease response of non-aggressive *A. brassicae* isolates on respective host differentials – [a] ABc-P01 (on *B. juncea* var. Rohini), [b-f] ABc-D06, ABc-P07, ABc-B01, ABc-H04 and ABc-Ho5 (on *B. juncea* var. PHR-01) respectively. (C) - Control-sterile distilled water, (NAG) - Non-Aggressive isolate, (HAG) - Highly Aggressive isolate and (NAG+HAG) - Non-Aggressive + Highly Aggressive isolate.

Table 4: Characterization of *A. brassicae* isolates on the basis of their aggressiveness on *B. juncea* var. PHR-01.

Group	Aggressiveness	Prevalent In	Isolates
I	Non-aggressive	Delhi	ABc-D06
		Pantnagar	ABc-P07, ABc-P12
		Bharatpur	ABc-B01
		Hisar	ABc-H04, ABc-H05
II	Low	Delhi	ABc-D07
		Ludhiana	ABc-L01, ABc-L10
		Bharatpur	ABc-B03
		Hisar	ABc-H03, ABc-H06
		Kangra	ABc-Kg01
III	Moderate	Delhi	ABc-D01, ABc-D02
		Pantnagar	ABc-P04, ABc-P05, ABc-P06, ABc-P08, ABc-P11
		Ludhiana	ABc-L07, ABc-L12, ABc-L19, ABc-L20
		Kangra	ABc-Kg02
		Kanpur	ABc-Kn01
IV	High	Pantnagar	ABc-P01, ABc-P09, ABc-P10
		Ludhiana	ABc-L02, ABc-L05, ABc-L08

26.3 and 3.7 % respectively.

Discussion

Thorough knowledge of the variability existing amongst the pathogen population and their response is the primary prerequisite for understanding any host-pathogen system. Moreover, it is also highly crucial in the process of breeding for resistance/ tolerance against a particular disease. A major lacuna in developing resistance/ tolerance against *Alternaria* blight disease of crucifers is the absence of sufficient documentation of pathogen behavior and its variability under different geographical locations. Identification and classification of *Alternaria* species as well as their pathotypes has been shown to be greatly influenced by the environmental conditions. In view of this, the present study was carried out with 55 *Alternaria* isolates procured and purified from eight states of north and north-west regions of India. Our earlier study reports the characterization of these isolates on the basis of their morphological, cultural, biochemical and molecular characteristics. Attempt was also made to correlate the total carbohydrate concentration of the isolates with the pathological response towards host; however, contradictory to the results obtained by Vishwanath & Kolte [20] consistent direct proportionality was not observed. Also consistent grouping with respect to all the characters studied could not be done because of the wide diversity obtained [3]. Pathological assay of the isolates in this study, on selected host differentials has shown significant variations among the isolates at inter and intra specific levels. Such a level of extensive variations indicates that the level of genetic variance in host resistance and pathogen virulence can strongly influence the population dynamics and equilibrium of the interacting species. The earlier studies on various host-pathogen systems [21], including *Alternaria-Brassica*, are restricted to single *Alternaria* species, with limited host differentials [2,22,5,6,10].

The present study reports the existence of pathological variations among the three most common *Alternaria* species pathogenic to *brassica*s viz., *A. brassicae*, *A. brassicicola* and *A. alternata* on host-differentials of *B. juncea* (four varieties) and *B. rapa* (two varieties) and also between the isolates of these species collected from different locations. The pathological response of the isolates was also found to vary with respect to different hosts. The isolates have shown varied response on the selected host differentials ranging from high to moderately aggressive to non-aggressive. Overall, *A. brassicae* and *A. brassicicola* isolates from Pantnagar have consistently shown high aggressiveness varying from 47-69 % and 51-77 % respectively. Although *B. juncea* var. Varuna is generally used as a susceptible check for *Alternaria* blight and White rust by many researchers, the present study shows that the pathological response varies from moderate to high and that all isolates are not highly aggressive on *B. juncea* var. Varuna.

In recent times, drastic variations in the pathogen behavior, increased disease severity and resistance to available fungicides have raised a doubt on the sustainability of chemical methods as sole strategy for disease control in *Brassica*s. In this changing scenario, biocontrol measures for disease management have gained considerable importance. One such method is generation of Induced Systemic Resistance (ISR) in plants i.e. activation of plant's natural defense mechanisms by prior inoculation with a non-aggressive pathogen, limited inoculum of the pathogen or its products. Reports have shown the utilization of microbial spore suspensions, culture filtrates and plant extracts resulting in reduction of *Alternaria* blight disease severity in vegetable and oilseed *brassica*s [23-26]. The reduction in the disease severity in all such cases is due to the resultant Pathogenesis-Related (PR) proteins released via Salicylic Acid (SA) / Jasmonic Acid (JA) pathways activated in response to the pathogen attack. Application of ISR as a disease control strategy demands for the identification of the non-aggressive isolates of the pathogen from the diverse gene pool. In our study, among all the isolates evaluated for their aggressiveness, non-aggressive behavior was observed on different host-differentials by ABc-P01 (on *B. juncea* var. Rohini), ABc-D06, ABc-P07, ABc-P12, ABc-B01, ABc-H04 (on *B. juncea* var. PHR-01), and ABc-H05 (on *B. juncea* var. PHR-01 and *B. rapa* var. YSH-401). These isolates were further tested for induction of systemic resistance. Out of the 7 non-aggressive isolates tested, five isolates (ABc-P01, ABc-D06, ABc-P07, ABc-B01 and ABc-H05) through the process of attenuated virulence were able to reduce the disease severity of corresponding highly aggressive isolates, thereby inducing systemic resistance in their respective hosts. These results support the findings of Vishwanath *et al.* [7] who reported the induction of resistance in PR-15 variety of mustard against virulent *A. brassicae* isolates A and C using avirulent *A. brassicae* isolate D.

To conclude, the *Alternaria* isolates purified from different *Brassica* species from varied regions in North and North-West India showed differential aggressiveness on selected host-differentials and a few *A. brassicae* isolates also induced systemic resistance against their highly aggressive counterparts. The availability of such pathotypes with varying degrees of pathogenicity/ aggressiveness towards different *Brassica* species and sub-species acts as an important base in identification, breeding and exploitation of durable resistance genotypes. Newer isolates/ isolates from other regions of India may be evaluated to identify the isolate capable of inducing systemic

resistance in a wider range of *B.juncea* varieties. The present study substantiates the possibility of deriving systemic resistance in *brassica*s against *Alternaria* blight via attenuated virulence. Integration of ISR-triggering non-aggressive pathotypes of the plant pathogens along with other disease control strategies could lead to an eco-friendly mode of attaining sustainable disease resistance in plants.

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