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Morpho-Physiological and Pathogenic Variability among *Alternaria alternata* Isolates causing Leaf Spot Disease of *Aloe vera*

K. Kannan*, S. Mohan and S. Malathi
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore.
Department of Plant Pathology, Agricultural College and Research Institute, Madurai.

Leaf spot disease of *Aloe vera* caused by *Alternaria alternata (Fr.) Keissler agg*. had high variability among different isolates. This study was conducted on pathogenicity, cultural and physiological (temperature and pH) characters. The disease incidence was maximum in Kottampatti of Madurai District (64.92 per cent) of Tamil Nadu, India followed by Sindhalachery of Theni District (58.83 per cent). Carrot dextrose medium favoured the highest mycelial growth of the pathogen both in solid (8.48cm) and liquid (2.08g/100ml) state. The thermal death point and pH for the growth of *A. alternata (Fr.)* was 47₀C and 5.5 pH respectively. The isolates of *A. alternata* differed in length, width and beak of conidia .

Key words: Leaf spot, *Aloe vera*, cultural characters, pH, solid and liquid media

Aloe vera (Syn: *Aloe barbadensis*) popularly known as Indian Aloe (or) Barbados Aloe is a perennial herb with fleshy and succulent leaves which have great medicinal value. Medicinal properties range from external burn treatments to relieve constipation by consumption. The most commonly reported use of *Aloe vera* gel is to speed the healing of burns, abrasions and other skin injuries. (Davis, 1997 and Radmilo, 2007). During 2006 *Aloe vera* production in Tamil Nadu was reduced due to the outbreak of leaf spot caused by *Alternaria alternata* / (Fr.) Keissler Agg. This disease was first reported by Kamalakannan *et al.* (2008) in Tamil Nadu, India. An understanding of the role of environmental conditions and its effect on infection and survival of the pathogen was necessary to develop cultural disease management practices (Sudarshan Rao, 1975). Therefore the objectives of this study included isolation, and purification of pathogenic fungus causing leaf spot of *Aloe vera* and determining optimal conditions for the mycelial growth of the fungus including pH, temperature and nutrient media.

Materials and Methods

A survey was conducted in different aloe growing areas of Tamil Nadu, India during the year 2009 to assess the leaf spot disease incidence. In each place three fields were selected at random, in each field 100 leaves were marked at random and disease grades were assigned as per the standard disease grade chart (TNAU, 1980). The Per cent Disease Incidence (PDI) was worked out by using McKinney (1923) formula *viz.,*

$$PDI = \frac{\text{Over all numerical ratings}}{\text{Total number of leaves observed X Maximum disease grade}} \text{ X } 100$$

*Corresponding author email: pathkannan@yahoo.com

Isolation and pathogenicity of fungus

The pathogen was isolated from the diseased tissues of *Aloe vera* by tissue segment method (Rangaswami, 1958) and stored at 4₀ C. The pathogenicity of the isolates was proved by following the method described by Mesta, (2006). The organism was re-isolated from these artificially infected leaves and the culture obtained was compared with the original culture for confirmation. Ten leaves were randomly selected from three replications of each isolate and disease grades were assigned as per the standard grade chart (TNAU, 1980). The Per cent Disease Incidence (PDI) was worked out by using McKinney (1923) formula as described earlier.

Morphological characters

Nine millimeter disc of *A. alternata* from the nine day old culture was placed at the center of Petri plate containing 20 ml of sterilized and solidified Potato Dextrose Agar (PDA) medium. The plates were incubated at room temperature (28 ± 2°C) for five days. The growth and morphological characters of the isolates *viz*., conidia size, shape, colour, septation, number of cells per conidium and beak length were observed and measured under microscope (magnification 45x) (Mesta, 2006).

Growth characters on solid media

The non-synthetic media like Potato dextrose agar, oat meal agar, malt extract agar, carrot dextrose agar, radish dextrose agar, beet root dextrose agar and the synthetic media like Richard's medium, Czapek (Dox) agar medium, modified Czapek (Dox) agar medium were used for assessing the growth of virulent isolate of *A. alternata* (Kamalalakshmi, 1996). The nine millimeter disc of the pathogen

was inoculated at the center of the plate containing solidified sterile medium. The plates were incubated at room temperature (28±2°C) and three replications were maintained in each medium to measure the radial growth of the mycelium after nine days of inoculation.

Growth characters on liquid media

In order to understand the variation among the isolates of *A. alternata* with respect to their growth in different liquid culture media, all the isolates of *A. alternata* were grown on potato dextrose broth and oat meal broth, Richard's broth, Czapek (Dox) broth, modified Czapex (Dox) broth, carrot dextrose broth, radish dextrose broth, beet root dextrose broth and malt extract in three replications. They were incubated at 27 ± 1$_o$ C for 10 days and the resulting growth of fungus was harvested and dried at 40 $_o$C for two days in hot air oven and the weights were recorded (Mesta, 2006).

Effect of pH and temperature on in vitro growth of A. alternata

The effect of pH on the growth of the pathogen was studied following the method described by Kiryu (1939) using Carrot Dextrose Agar medium. The effect of temperature on growth of the pathogen was studied using spore suspension (5 x 10$_5$ spores ml$_{-1}$) of *A. alternata* by following the method described by Kamalalakshmi (1996).

Results and Discussion

Natural disease incidence and *in vitro* pathogenic variability

Incidence of *A. alternata* leaf spot was maximum(64.92 %) in Kottampatti (Madurai district) followed by Sindhalachery (Theni district) (58.83%) (Table 1). The sudden increase in disease incidence might be due continuous maintenance of *Aloe vera* as perennial shrub in the fields without following crop rotation system. Continuous cultivation of any crop over the season and years will build up inoculum level to such an extent that the epidemic will become a common phenomenon (Chaube and Singh, 2001). Under *in vitro* conditions artificially inoculated leaves showed black spots with concentric rings; in due course the spots increased in size and turned as sunken lesions. The lesions gradually exhibited a grayish tint at the center surrounded by a brown margin. The maximum disease incidence was recorded in Kottampatti isolate (I_6) inoculated plants (85. 73 PDI) whereas minimum disease intensity was recorded in I_2 from Chekkanoorani of Madurai district (37.74 PDI) (Table 1). This finding was in corroboration with Adachi *et al*. (1994) and Weir *et al.* (1998) who suggested the existence of multiple

Table 1. Effect of different isolates of *A. alternata* in Tamil Nadu in causing leaf spot severity in *Aloe vera* both under *in vivo* and *in vitro* condition

Isolate	Place	Natural disease incidence (%) *	Disease incidence in *vitro* (%)
I_1	Alanganallur (Madurai)	38.07 (38.09)**	40.17 (37.49)**
I_2	Chekkanoorani (Madurai)	25.97 (30.63)	37.74 (37.9)
I_3	Markaiyankottai (Theni)	44.51 (41.84)	52.85 (48.55)
I_4	Nattarasankottai (Sivagangai)	43.51 (41.27)	59.79 (50.86)
I_5	Singampunari (Sivagangai)	56.61 (48.79)	61.07 (56.31)
I_6	Kottampatti (Madurai)	64.92 (61.37)	85.73 (76.04)
I_7	Killikulam (Tirunelveli)	43.62 (41.33)	66.39 (42.93)
I_8	Sindhalachery (Theni)	58.83 (52.12)	71.52 (64.15)
I_9	Pulipatti (Madurai)	31.30 (34.01)	65.49 (54.02)
I_{10}	Palladam (Tirupur)	34.41 (35.91)	55.19 (46.12)
	C. D (P = 0.05%)	1.46	1.28

* Mean of three replications .

**Values in the parentheses are arc sine transformed values

biotypes within species as reason for the pathogenic variability.

Morphological variability among A. alternata isolates

Morphological characters are important tools in identification and classification of the fungus. In the present study, spore size, septation of conidiophore conidia as well as shape of conidia were used to identify the fungus. The characteristics of the fungus were compared with *Alternaria* species reported on other host and characters agreed very closely with those of *A. alternata* (Fr.) Keissler with minor variation in shape dimension which may be either, due to host or environmental factor and fall within the limits for species. In all the ten isolates, the conidia were obclavate shape and brown in colour. The length of the conidia varied from 31.75 to 63.34 µm. The longest conidia (63.34 mm) were observed in *A. alternata* isolate I_6 from Kottampatti and was very short in *A. alternata* isolate I_4 from Sivagangai (31.75

Table 2. Morphological and conidial characters of *A. alternata* isolates in *Aloe vera* collected from different locations of Tamil Nadu

Isolate	Shape of conidia	Colour of conidia	Colour of mycelium	Length (µm) *	Width(µm) *	Beak length (µm) *	No. of cells*	Unbeaked conidia (%)*
I_1	Obclavate	Brown	Grey	60.05	17.25	7.13	2-7	0
I_2	Obclavate	Brown	Grey	35.17	15.2	5.6	2-6	1
I_3	Obclavate	Brown	Black	57.45	16.2	6.07	3-8	3
I_4	Obclavate	Brown	Grey	31.75	13.7	4.75	3-5	4
I_5	Obclavate	Brown	Grey	59.17	16.8	6.87	2-5	2
I_6	Obclavate	Brown	Grey	63.34	18.05	8.03	3-9	0
I_7	Obclavate	Brown	Black	61.28	17.6	7.28	2-6	3
I_8	Obclavate	Brown	Grey	43.3	14.28	5.21	3-9	0
I_9	Obclavate	Brown	Grey	58.29	16.45	6.14	3-8	5
I_{10}	Obclavate	Brown	Grey	46.28	15.85	5.92	3-8	6
C. D (P = 0.05%)				0.30	0.06	0.49	-	-

* Mean of three replications of twenty number of conidia observed under light microscope at 45x magnification

mm). The width of the conidia varied from 13.70 – 18.05 µm. Generally all the isolates produced both beaked and unbeaked conidia. The beak length of conidia varied from 4.75 to 8.03 µm. The isolate I_6 had the longest beak (8.03 mm) while it was short in I_4 (4.75 mm) The number of cells in each conidium varied from 2 to 9 (Table 2). These morphological characters were in agreement with the descriptions given by Kamalakannan *et al.* (2008), Muthulakshmi (1990) and Kamalalakhsmi (1996) .

Cultural characters

Effect of solid and liquid media on mycelial growth of A. alternata isolates.

Growth of *A. alternata* on different media and cultural characteristics are often taken as general guidelines for differentiating the strains/ isolates of the fungal pathogen. The mean diameter of mycelial growth of the pathogen was significantly maximum in carrot agar medium (8.48cm) while it was less in beet root agar medium (7.08cm). Among the isolates tested, isolate I_6 collected from Kottampatti had maximum mean mycelial growth of 8.31cm whereas the minimum mean mycelial growth was recorded in I_5 collected from Singampunari (7.46cm) . The isolate I_6 recorded higher mycelial growth on carrot agar medium, indicating that this isolate is fast growing, highly virulent and potential to cause the

Table 3. Influence of different semisynthetic media on the growth of *A. alternata* isolates collected from *Aloe vera* growing areas of Tamil Nadu

Isolate	Diameter of mycelial growth(cm)*									
	PDA	OMA	C(D)A	RA	MEA	MC(D)A	CDA	RDA	BRA	Mean
I_1	8.23	7.00	7.41	7.21	8.37	6.92	8.73	8.13	7.02	7.67
I_2	8.42	7.21	8.17	7.34	7.61	6.73	8.27	8.32	7.32	7.71
I_3	7.91	8.01	7.92	6.98	8.43	6.82	8.65	7.91	7.64	7.84
I_4	7.60	7.48	7.63	6.81	8.64	8.01	8.13	7.82	7.38	7.73
I_5	7.14	8.02	7.81	7.02	7.34	7.31	7.91	8.14	6.42	7.46
I_6	8.81	8.63	8.57	7.64	8.68	8.28	8.90	8.43	7.32	8.31
I_7	7.68	8.13	8.00	7.63	7.62	7.61	8.01	7.63	5.96	7.59
I_8	8.02	7.92	7.03	6.93	8.10	7.73	8.73	8.27	6.72	7.73
I_9	8.11	7.81	7.68	7.63	7.31	6.89	8.84	7.9	6.98	7.68
I_{10}	7.81	8.30	7.13	7.73	8.31	8.02	8.59	8.5	8.01	8.04
Mean	7.97	7.85	7.74	7.29	8.04	7.43	8.48	8.10	7.08	-

* Mean of three replications (for each replication ten plates were maintained)

C. D (P = 0.05%)

Isolates - 0.20 Media - 0.19 Isolates X Media - 0.62

PDA- Potato Dextrose Agar, OMA- Oat Meal Agar, C(D)A- Czapek(Dox) Agar, RA- Richard's Agar, MEA- Malt Extract Agar, M C(D)A-Modified Czapek (Dox) Agar, CDA- Carrot Dextrose Agar, RDA- Radish Dextrose Agar, BRA-Beet Root Agar.

The following are the isolates and their collected places,

I_1- Alanganallur, I_2-Chekkanoorani, I_3-Markaiyankottai, I_4-Nattarasankottai, I_5-Singampunari, I_6-Kottampatti, I_7-Killikulam , I_8-Sindhalachery, I_9-Pulipatti,I_{10}-Palladam.

Table 4. Influence of different nutrient broth on growth of *A. alternata* isolates collected from *Aloe vera* growing areas of Tamil Nadu

Isolates	Dry weight of mycelial growth(g)*									
	PDB	OMB	C(D)B	CDB	RB	MC(D)B	RDB	BDB	MEB	Mean
I_1	0.89	0.41	0.89	2.10	0.43	0.72	1.03	0.40	0.94	0.88
I_2	0.96	0.62	0.81	2.12	0.85	0.85	1.07	0.64	0.96	0.98
I_3	0.81	0.72	1.10	1.98	0.91	0.68	0.92	0.61	1.12	0.98
I_4	0.69	0.61	0.91	1.72	0.56	0.72	0.87	0.72	1.00	0.86
I_5	0.84	0.51	0.82	2.01	0.71	0.71	0.94	0.58	1.06	0.91
I_6	0.72	0.81	1.21	2.31	0.84	0.69	1.21	0.62	1.07	1.05
I_7	0.93	0.72	0.72	2.07	0.82	0.82	1.01	0.71	0.89	0.96
I_8	1.01	0.65	0.89	2.01	0.81	0.84	1.08	0.58	0.82	0.97
I_9	0.74	0.72	0.97	2.12	0.80	0.80	0.99	0.49	0.85	0.94
I_{10}	0.63	0.82	1.03	2.08	0.81	0.81	0.97	0.57	0.83	0.95
Mean	0.82	0.66	0.93	2.08	0.75	0.75	1.01	0.59	0.95	-

* Mean of three replications (for each replication ten plates were maintained)

C. D (P = 0.05%)

Isolates - 0.028 Broth - 0.026 Isolates X Broth - 0.084

PDB- Potato Dextrose Broth, OMB- Oat Meal Broth, C(D)B- Czapek(Dox) Broth, RB- Richard's Broth, MEB- Malt Extract Broth, M C(D) B- Modified Czapek (Dox) Broth, CDB- Carrot Dextrose Broth , RDB- Radish Dextrose Broth, BRB-Beet Root Broth.

The following are the isolates and their collected places,

I_1- Alanganallur, I_2-Chekkanoorani, I_3-Markaiyankottai, I_4-Nattarasankottai, I_5-Singampunari, I_6-Kottampatti, I_7-Killikulam , I_8-Sindhalachery, I_9-Pulipatti,I_{10}-Palladam

disease (Table 3). Among the liquid media tested, the highest mean mycelial dry weight of 2.08g/100 ml was recorded in carrot dextrose broth while beet root dextrose broth had least mean mycelial dry weight (0.59g/100ml) (Table 4). Maximum mycelial growth of the pathogen on Carrot medium (both in solid and liquid state) was due to the presence of vitamins and other favourite nutrients in Carrot which are necessary for fungal growth and metabolism (Dubey, 2005). Nutrients in Carrot, support the maximum growth indicating that the pathogen utilize readily the available nutrients than highly complex compounds.

Physiological variability

Growth of A. alternata isolates at different pH levels

Change in pH plays an important role in hydrogen ion concentration which is essential for the growth of fungi. The maximum mean mycelial growth (86.57 mm) was observed in pH 5.5 (Table 5), whereas the minimum mycelial growth was observed in pH

Table 5. Effect of different pH levels on the growth of *A. alternata* isolates collected from different *Aloe vera* growing areas of Tamil Nadu

Isolates	Diameter of mycelial growth(mm)*									
	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	Mean(cm)
I_1	58.49	71.67	80.01	87.13	71.07	61.07	68.37	61.08	62.08	69.00
I_2	61.03	72.68	81.13	89.17	80.41	73.01	64.82	63.07	54.12	71.05
I_3	62.61	68.01	82.16	88.13	81.04	68.13	69.18	62.08	61.13	71.39
I_4	67.08	63.01	84.07	86.10	76.13	64.03	61.03	60.82	59.64	69.10
I_5	69.03	64.72	80.13	84.12	74.08	69.18	70.01	59.07	56.34	69.63
I_6	68.14	67.13	81.13	89.17	82.61	76.01	72.64	68.13	67.21	74.69
I_7	59.13	70.14	78.64	86.34	78.04	70.17	69.72	61.08	67.13	71.15
I_8	71.03	73.16	76.12	88.16	80.17	71.78	68.19	64.08	50.31	65.44
I_9	70.61	69.11	79.18	80.17	81.07	72.18	67.16	65.12	56.12	71.19
I_{10}	60.13	63.10	80.18	87.19	79.16	73.18	70.17	61.14	58.61	70.32
Mean	59.328	68.273	80.275	86.568	78.378	69.874	68.129	62.567	59.269	--

* Mean of three replications (for each replication ten plates were maintained) C. D (P = 0.05%)

Isolates - 0.15 pH - 0.11 Isolates X pH - 0.37

The following are the isolates and their collected places,

I_1- Alanganallur, I_2-Chekkanoorani, I_3-Markaiyankottai, I_4-Nattarasankottai, I_5-Singampunari, I_6-Kottampatti, I_7-Killikulam , I_8-Sindhalachery, I_9-Pulipatti,I_{10}-Palladam.

8.0 (59.27 mm). The isolates I_6 and I_2 recorded the maximum mycelial growth in pH 5.5 (89.17 mm). This finding is in line with the work of Saeed *et al*. (1995) who observed that isolates of *A. alternata* grew best at pH 5.5. The present study was also in conformity with the reports of Ramamohana Rao and Vijayalakshmi (2000) who observed good growth of *Alternaria* species between pH 4.0 and 6.0. The reason may be that isolates collected from a particular location might have adjusted themselves according to condition prevailing in that area and might have adopted to a particular pH for growth and sporulation (Mehta *et al.,* 2005).

Thermal death point of A. alternata isolates

Temperature is the most important environmental factor regulating vegetative and reproductive activity of the fungi The conidia of all ten isolates of *A. alternata* germinated up to 45°C. The conidia of the isolates I_4, and I_7 germinated at 45°C, but they failed to germinate at 46°C indicating as a thermal death point, while the remaining had thermal death point

Table 6. Thermal death point of *A. alternata* isolates collected from *Aloe vera* growing areas of Tamil Nadu

Isolate	41^{0}C	42^{0}C	43^{0}C	44^{0}C	45^{0}C	46^{0}C	47^{0}C	48^{0}C	49^{0}C	50^{0}C
I_1	+	+	+	+	+	+	-	-	-	-
I_2	+	+	+	+	+	+	-	-	-	-
I_3	+	+	+	+	+	+	-	-	-	-
I_4	+	+	+	+	+	-	-	-	-	-
I_5	+	+	+	+	+	+	-	-	-	-
I_6	+	+	+	+	+	+	-	-	-	-
I_7	+	+	+	+	+	-	-	-	-	-
I_8	+	+	+	+	+	+	-	-	-	-
I_9	+	+	+	+	+	+	-	-	-	-
I_{10}	+	+	+	+	+	+	-	-	-	-

\+ Presence of the mycelial growth.

Absence of the mycelial growth

The following are the isolates and their collected places,

I_1- Alanganallur, I_2-Chekkanoorani, I_3-Markaiyankottai, I_4-Nattarasankottai, I_5-Singampunari, I_6-Kottampatti, I_7-Killikulam , I_8-Sindhalachery, I_9-Pulipatti,I_{10}-Palladam.

at 47°C (Table 6). The thermal death point of the isolates of *A. alternata* differed with a narrow range of 46-47^{o}C which is in accordance with the finding of Karthikeyan (1999). Agrios (2006), reported that the temperature above 46$_o$C affect the hyphal tip elongation and appressorial formation.Paea Aloevera the study indicated that carrot dextrose agar, pH 5.5 and a temperature up to 45°C were favourable for the growth of *A. alternata* from However the virulence varied among the isolates in causing disease severity.

References

Adachi, Y. and Tsuge, T. 1994. Co infection by different isolates of *Alternaria alternata* in single black spot lesions of Japanese pear leaves. *Phytopathol.,* **84**: 447 - 451.

Agrios, G.N. 2006. Plant Pathology. Academic Press, New Delhi. 90p.

Chaube, H.S. and Singh, R. 2001. Introductory Plant Pathology. International Book Distributing Company, Lucknow. 132p.

Davis Robert, H. 1997. "*Aloe vera*: A Scientific Approach". Vantage Press, New York, 1st Edition. 35, 36,109-111.

Dubey, H.C. 2005. An introduction to fungi. Vikas publishing house. Jangpura, New Delhi. 446p.

Kamalakannan, A., Gopalakrishnan, C., Renuka, R., Kalpana, K., Ladha Lakshmi, D. and Valluvaparidasan, V. 2008. First report of *Alternaria alternata* causing leaf spot on *Aloe barbadensis* in India. *Australian Plant Disease Notes*, **3:** 110-111.

Kamalalakhsmi, C. 1996. Studies on leaf blight disease of Jathimalli *Jasminum grandiflorum* L. incited by *A. alternata* (Fr.) Keissler.agg. M.Sc. (Ag.) Thesis, Tamil Nadu Agric.Univ., Coimbatore, India. 199p.

Karthikeyan, M. 1999. Studies on onion (*Allium cepa* var *aggregatum* L.) leaf blight caused by *Alternaria palandui* Ayyangar. M.Sc., (Ag.) Thesis**,** Tamil Nadu Agric. Univ., Coimbatore, India. 120p.

Kiryu, T. 1939. Studies on physiological properties of *Ceratostomea paradoxa*. Jaiwan province sugarcane experiments station report, **6:** 21-27.

Mckinney, H.H. 1923. A new system of grading plant diseases. *J. Agric. Res*., **26**: 195-218.

Mehta, N., Khurana A.K. and Sangwan, M.S. 2005. **In:** *Integrated Plant Disease Management.* (Eds.) Sharma, R.C. and Sharma, J.N), Scientific Publishers (India) Jodhpur, 295-303p.

Mesta, R.K. 2006**.** Epidemiology and management of *Alternaria* blight of sunflower caused by *Alternaria helianthi* (hansf.) Tubaki and Nishihara, Ph.D., Thesis, Univ. Agric. Sci., Dharwad-5, India. 159p.

Muthulakshmi, P. 1990. Studies on fruit rot of chillies (*Capsicum annum* L.) caused by *Alternaria tenuis* Nees. M.Sc., (Ag.) Thesis**,** Tamil Nadu Agric. Univ., Coimbatore, India. 139p.

Radmilo, Anicic, "Aloe", *Aloe vera* Community of Canada. 2007. *http://www.Aloeveracanada.ca/about_av.html*

Ramamohana Rao, N. and Vijayalakshmi, M. 2000. Studies on *Alternaria sesami* pathogenic to sesame. *Microbiol. Res.,* **155:** 129-131

Rangaswami, G. 1958. An agar blocks technique for isolating soil micro organisms with special reference to pythiaceous fungi. *Sci. and Cul.,* **24:** 85.

Saeed, M.A., Ahamed, M. and Khan, H.A. 1995. Effect of different media, temperature, and pH levels, nitrogen and carbon sources on the growth of *Alternaria* spp. *Pakistan J. Phytopath.,* **7:** 210-211.

Sudarshan Rao, A.N. 1975. Survey, surveillance and forecast in plant protection. *Kissan World.*, **2:** 43 – 44

TNAU- (Tamil Nadu Agricultural University). 1980. Score Chart for Crop Diseases, Coimbatore, India. 56p.

Weir, T.L., Huff, D.R., Christ, B.J. and Romaine, C.P. 1998. RAPD-PCR analysis of genetic variation among isolates of *Alternaria solani* and *A*. *alternata* from potato and tomato. *Mycologia*. **90**: 813-821.

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