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Biochemical changes during flowering in *Citrus* species

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ABSTRACT

The present experiment was conducted during 2017- 2018 with the objective to understand the relationship of seasonal changes in biochemical traits with the flowering behaviour of acid limes (Pusa Udit, Pusa Abhinav and ALC-29), lemons (Konkan Seedless, Kagzi Kalan and Hill lemon) and sweet oranges (Pusa Sharad, Pusa Round and Mosambi). Sweet oranges and Hill lemon expressed flowering once (February to March), and lemons and Acid limes bloomed almost round the year. In all lemons (except Hill lemon) and limes, protein level ≥ 9.30 mg g^{-1} FW promoted the flowering, however, it was ≥ 19.3 mg g^{-1} FW in Hill lemon and sweet oranges. In lemons and limes, superoxide dismutase (SOD) activity ≥ 9.88 Unit mg^{-1} min^{-1} TSP expressed the association with flowering except Pusa Abhinav lime. Hill lemon and sweet oranges bloomed at the activity of SOD ≥ 13.77 Unit mg^{-1} min^{-1} TSP. The catalase (CAT) activity did not follow a systematic pattern in most of the cultivars. In limes and lemons (except Hill lemon), the plants showing POX activity ≥ 17.77 µmol min^{-1} mg^{-1} total soluble proteins (TSP) showed flowering, while in rest of the genotypes, its level was ≥ 52.45 µmol min^{-1} mg^{-1} TSP to initiate the flowering. In most of the limes and lemons during August to February, ascorbate peroxidase (APOX) activity ≥ 0.20 µmol min^{-1} mg^{-1} TSP favoured the flowering, however, in Hill lemon and sweet oranges, it was ≥ 0.220 µmol min^{-1} mg^{-1} TSP. The lower activity of O_2^- (0.91-1.76 µmol g^{-1} FW) between August to January in limes and lemons (except Hill lemon) promoted the flowering.

Key words: Superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, free radicles.

INTRODUCTION

Flowering is an essential phenomenon in the life cycle of higher plants, being the central process of species reproduction. Flowering of tree crops is highly complex process involving many developmental stages. Generally, the timing of flowering is regulated by autonomous or environmental factors. Citrus (*Citrus* species.) comprising of a variety of crops including sweet orange, mandarin, grapefruit, lemon, lime etc. are grown worldwide. Citrus exhibits wide variations in flowering and fruiting due to its strong dependency on environment. In citrus, the bud break occurs in spring either as a result of completion of winter chilling, or as a result of drought stress for at least 30-35 days (Srivastava *et al*., 18). After the fulfilment of low temperature (chilling) requirements, the initiation of both leafing and floral development begins (Young and Werner, 21).

Various H_2O_2 scavenging enzymes and cell wall bound (CWB) G-POD have shown link with flowering in lemon (Kasraoui *et al*., 10). The changes in antioxidant enzymes (CAT, POD, APX) and sulfhydryl compounds may provide defensive mechanism against inhibitory compounds generated during the course of this critical period viz., flowering (Mondal *et al.,* 12). Limited information is available on the activity of biochemical compounds in citrus during bud development. Thus, the present study aimed to study the changes in the activity of biochemical compounds in developing buds and neighbouring leaves leading to reproductive expression in citrus species having diverse flowering behaviour.

MATERIALS AND METHODS

The experiment was conducted in the Experimental Orchard of the Division of Fruits and Horticultural Technology, IARI, New Delhi during August, 2017 to March, 2018 on three *Citrus* species with three genotypes of each (Table 1.), for studying the biochemicals trend and their association with the flowering behaviour. Four shoots on each plant were selected and tagged, and the following data on various biochemical constituents were recorded at monthly interval. The experiment was laid out in Randomized Block Design and replicated thrice. The data were recorded at the appearance of tiny white tip of flower bud.

For biochemical parameters estimation, the fresh fully expanded leaves were collected in the morning in ice box and brought to the laboratory. The leaves were washed in tap water followed by double-distilled water. The cleaned leaf sample (1.0 g) was homogenized in pre-chilled mortar and pestle with liquid nitrogen to prevent protolytic activity followed by adding 10 ml of chilled phosphate buffer (100 mM; pH

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Table 1. *Citrus* species tested in the study.

S. No.	Species	Genotypes
1.	*C. limon* (L.) Burm	Kagzi Kalan, Konkan Seedless and Hill lemon
2.	*C. aurantifolia* (Christ.) Swingle	Pusa Udit, Pusa Abhinav and ALC-29
3.	*C. sinensis* (L.) Osbeck	Mosambi, Pusa Round and Pusa Sharad

7.5) containing 0.5 mM EDTA in case of superoxide dismutase, catalase, and peroxidase, and 1.0 mM ascorbic acid in case of ascorbate peroxidase. The homogenate was collected in oak-ridge tubes and centrifuged at 15,000 × g for 20 min at 4^0C. The supernatant so obtained was strained through two layers of muslin cloth and stored in refrigerator (-20^0C). This supernatant was used as extract for the estimation of following antioxidant enzymes.

The activity of superoxide dismutase (SOD) in leaf sample was determined according to method outlined by Dhindsa *et al.* (8). The catalase (CAT) assay was based on the absorbance of hydrogen peroxide ($H_2O_{2)}$ at 240 nm in UV-range (Aebi, 2). The method suggested by Castillo *et al*. (5) was used to estimate peroxidase (POX) activity in leaf sample. Ascorbate peroxidase (APOX) enzyme was assayed according to the method outlined by Nakano and Asada (16). The total superoxide content was assayed according to the method described by Chaitanya and Naithani (6). Soluble protein content of the enzyme extract was estimated using Bradford protein assay (Bradford, 4). The data were analysed statistically using two-way analysis of variance, followed by Tukey's Honest Significant Difference (HSD) test available in SAS Software Version 9.3 (SAS Institute, Cary, NC, USA). *P* values ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

There was huge variation in the flowering behaviour of the species included in the present study. From September onwards, lime (Pusa Udit, Pusa Abhinav except October and ALC-29) and Lemon (Konkan Seedless and Kagzi Kalan) exhibited regular flowering, however, Hill lemon and all the varieties of sweet orange could blom once in a year i.e. January-February. The genotypes of *Citrus* species were found to have significant difference in the activity of SOD throughout the period of experimentation (Table 2). In present study, the activity of SOD was quite low initially in all the genotypes which increased rapidly between September to October, then followed a gradual upward trend reaching at peak in January, and declined thereafter. However, in Kagzi Kalan and Hill lemon, the activity of SOD declined initially, and then followed the similar trend as shown by the other genotypes. The highest activity of SOD in the month of August, September and March was recorded in the leaves of Kazi Kalan (13.16 Unit mg^{-1} min^{-1} TSP) and Konkan Seedless (15.81 Unit mg^{-1} min^{-1} TSP) lemons and ALC-29 lime (20.45-Unit mg^{-1} min^{-1} TSP), respectively. In general, Konkan Seedless and Kagzi Kalan lemons maintained the higher level of SOD activity during November to January. In general, limes and lemons (except Hill lemon) responded to flowering at relatively lower level of SOD that sweet orange and Hill Lemon. In lemons and limes, SOD activity ≥ 9.88 Unit mg^{-1} min^{-1} TSP has been found to be associated with the flowering. Hill lemon and sweet oranges showed flowering at the activity

Table 2. Seasonal variation in the superoxide dismutase (SOD) activity (Units mg^{-1} min^{-1} TSP) of citrus genotypes**.**

Species	Genotype	Month							
		Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar
C. aurantifolia	Pusa Udit	9.97^{d}	9.88^{e}	17.14^{b}	18.46^{bac}	20.21^{bac}	23.56^{cb}	22.14^{a}	17.17^{c}
	Pusa Abhinav	7.67^{f}	10.57^{d}	16.08^{d}	18.72^{bac}	20.56^{ba}	23.42^{cb}	21.35^{ba}	19.48^{b}
	ALC-29	12.26^{b}	14.08^{b}	18.15^{a}	18.86^{bac}	20.65^{ba}	23.74^{b}	21.14^{b}	20.45^{a}
C. limon	Konkan Seedless	11.74^{c}	15.81^{a}	17.95^{a}	19.15^{a}	20.90^{a}	24.95^{a}	19.23^{c}	14.89^{d}
	Kagzi Kalan	13.16^{a}	12.04^{c}	18.50^{a}	18.97^{ba}	21.02^{a}	25.02^{a}	19.55^{c}	16.85^{c}
	Hill Lemon	11.89^{cb}	10.18^{ed}	16.90^{cb}	18.12^{bc}	19.45^{c}	22.01^{ed}	14.14^{e}	14.01^{e}
C. sinensis	Pusa Sharad	7.67^{f}	10.30^{ed}	17.04^{cb}	18.23^{bac}	20.01^{bc}	21.56^{ed}	13.77^{e}	13.24^{f}
	Pusa Round	8.18^{e}	11.75^{c}	16.35^{cd}	17.90^{dc}	19.85^{bc}	22.45^{cd}	15.29^{d}	14.89^{d}
	Mosambi	6.99^{g}	9.36^{f}	16.39^{cbd}	17.00^{d}	19.56^{c}	21.23^{e}	14.37^{ed}	13.97^{e}

Values are means (n=3). Mean values in each citrus cultivar followed by different lower-case letters were significantly different at $P \leq 0.05$ by Tukey's HSD test.

of SOD ≥ 13.77 Unit mg^{-1} min^{-1} TSP. The results obtained in the present investigation are found to be in close conformity with the studies of Wang *et al*. (20).

The activity of POX was low initially, remained static between August to December, thereafter increased rapidly till February and declined with the approach of last month of sampling *i.e.,* March (Table 3). In all the genotypes of lime and lemon (except Hill lemon), the plants showing POX activity ≥ 17.77 µmol min^{-1} mg^{-1} TSP showed flowering, while in rest of the genotypes, its level was ≥ 52.45 µmol min^{-1} mg^{-1} TSP to express the flowering. This result is in conformity with the earlier findings of Bernal *et al*. (3) wherein flowering was found to be associated with an increase in POD activity in the leaves. Monerri and Guardiola (13) showed that changes in peroxidase activity occurred concomitantly with floral transition in *Citrus unshiu*.

The activity of APOX initially increased till October and declined thereafter in November, rose again to a peak in January (all limes and Konkan Seedlessa and Kagzi Kalan lemons) and February (Hill lemon and all sweet oranges), and their lowest value recorded on the last date of sampling (Table 4). In general, continuously bearing varieties of lemon and lime could respond particularly for flowering at relatively lower level of APOX activity over single bearing varieties. In most of the lime and lemon genotypes during August to February, APOX activity ≥ 0.200 µmol min^{-1} mg^{-1} TSP favoured the flowering. However, in Hill lemon and sweet orange, the similar response was noticed in February when its APOX activity was ≥ 0.220 µmol min^{-1} mg^{-1} TSP. Similar results with increased activity of APOX during flowering has also been reported by Kasraoui *et al*. (10) in lemon.

Table 3. Seasonal variation in the peroxidase (POX) activity (µmol min^{-1} mg^{-1} TSP) of citrus genotypes.

Species	Genotype	Month							
		Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar
C. aurantifolia	Pusa Udit	10.73^{f}	18.04^{c}	19.37^{c}	20.32^{ba}	28.85^{a}	48.65^{a}	63.37^{ed}	55.45^{cde}
	Pusa Abhinav	11.39^{e}	17.47^{c}	13.71^{g}	18.75^{d}	26.63^{b}	42.65^{c}	65.94^{bcd}	57.14^{cb}
	ALC-29	18.75^{b}	20.40^{b}	10.45^{h}	19.66^{bc}	22.66^{d}	44.52^{cb}	61.41^{e}	53.56^{de}
C. limon	Konkan Seedless	11.62^{e}	19.89^{b}	22.24^{b}	19.08^{dc}	23.31^{d}	42.78^{c}	66.92^{bc}	58.31^{cb}
	Kagzi Kalan	25.94^{a}	23.33^{a}	24.66^{a}	20.62^{a}	25.09^{c}	45.95^{b}	68.26^{ba}	59.56^{b}
	Hill Lemon	14.42^{c}	15.39^{e}	16.32^{e}	16.66^{e}	15.59^{g}	34.95^{d}	70.97^{a}	62.75^{a}
C. sinensis	Pusa Sharad	11.57^{e}	15.86^{ed}	16.61^{e}	15.34^{f}	18.64^{e}	35.02^{d}	66.21^{bcd}	56.12^{cd}
	Pusa Round	13.39^{d}	16.30^{d}	15.81^{f}	14.67^{f}	17.42^{f}	34.68^{d}	64.48^{ecd}	56.1^{cd}
	Mosambi	14.74^{c}	13.19^{f}	17.59^{d}	15.25^{f}	16.09^{g}	33.78^{d}	68.05^{ba}	52.45^{e}

Values are means (n=3). Mean values in each citrus cultivar followed by different lower-case letters were significantly different at $P \leq 0.05$ by Tukey's HSD test.

Table 4. Seasonal variation in the ascorbate peroxidase (APOX) activity (µmol min^{-1} mg^{-1} TSP) of citrus genotypes.

Species	Genotype	Month							
		Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar
C. aurantifolia	Pusa Udit	0.180^{c}	0.210^{c}	0.240^{ba}	0.170^{d}	0.230^{b}	0.280^{c}	0.230^{de}	0.080^{e}
	Pusa Abhinav	0.150^{f}	0.220^{b}	0.210^{c}	0.180^{c}	0.210^{d}	0.300^{b}	0.220^{e}	0.090^{d}
	ALC-29	0.200^{b}	0.230^{a}	0.230^{b}	0.180^{c}	0.200^{e}	0.270^{d}	0.240^{dc}	0.070^{f}
C. limon	Konkan Seedless	0.170^{d}	0.230^{a}	0.240^{ba}	0.190^{b}	0.220^{c}	0.310^{a}	0.250^{a}	0.100^{dc}
	Kagzi Kalan	0.210^{a}	0.200^{d}	0.250^{a}	0.210^{a}	0.240^{a}	0.280^{c}	0.240^{bc}	0.090^{d}
	Hill Lemon	0.160^{e}	0.170^{f}	0.190^{d}	0.140^{g}	0.180^{g}	0.240^{e}	0.220^{e}	0.110^{b}
C. sinensis	Pusa Sharad	0.150^{f}	0.160^{g}	0.210^{c}	0.160^{e}	0.190^{f}	0.210^{f}	0.250^{ba}	0.140^{a}
	Pusa Round	0.140^{g}	0.170^{f}	0.230^{b}	0.150^{f}	0.180^{g}	0.200^{g}	0.260^{a}	0.110^{b}
	Mosambi	0.130^{h}	0.180^{e}	0.200^{dc}	0.190^{b}	0.210^{d}	0.210^{f}	0.240^{bc}	0.090^{d}

Values are means (n=3). Mean values in each citrus cultivar followed by different lower-case letters were significantly different at $P \leq 0.05$ by Tukey's HSD test.

The activity of CAT enzyme exhibited the significant variation in different citrus genotypes, while measured at monthly intervals between August to March. The CAT activity did not follow a systematic pattern which fluctuated between September to March, however, in Pusa Abhinav lime, the activity of CAT followed a reverse trend between September to November (Table 5). Abassi *et al*. (1) evaluating Red Spur Delicious flower bud development, reported that changes in catalase and peroxidase activities were related to growth and development of the apple flower.

During phase transition from vegetative to reproductive, the cellular antioxidant status of plants tends to improve, suggesting the exposure of plants to stressful conditions favour for the onset of reproductive growth (Wada and Takeno, 19). The antioxidant enzymes are one of the important biochemical factors for transition of vegetative growth to flowering (Gohari *et al*., 9). Moradi *et al*. (15) in pomegranate reported the increase in antioxidant enzymes by flowering inducing growth regulator (paclobutrazol) under stress condition.

Various citrus genotypes expressed significant difference for the level of protein content in this study, which was initially low, remained static in Pusa Udit and Pusa Abhinav limes, Hill lemon and all sweet orange genotypes till January, however, it rose slightly in Konkan Seedless and Kagzi Kalan lemons till January. A sharp increase in the content of protein was noticed between January to February, and declined thereafter. In all lemons (except Hill lemon) and limes, protein level ≥ 9.30 mg g^{-1} FW promoted the flowering, however, in Hill lemon and sweet oranges, the protein content ≥ 19.3 mg g^{-1} FW proved effective to induced the flowering (Table 6). During the process of floral initiation, a sequence of biochemical

Table 5. Seasonal variation in the catalase (CAT) activity (µmol min^{-1} mg^{-1} TSP) of citrus genotypes.

Species	Genotype	Month							
		Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar
C. aurantifolia	Pusa Udit	12.45^{f}	17.88^{c}	24.12^{b}	18.11^{c}	25.03^{dc}	12.02^{a}	28.67^{c}	23.56^{b}
	Pusa Abhinav	13.58^{e}	19.57^{b}	15.08^{f}	19.46^{b}	26.90^{ba}	10.01^{c}	26.98^{d}	21.45^{c}
	ALC-29	17.26^{b}	18.08^{c}	25.38^{a}	20.42^{a}	24.45^{de}	9.85^{c}	24.14^{e}	20.45^{dc}
C. limon	Konkan Seedless	16.74^{b}	23.81^{a}	23.28^{cbd}	18.75^{cb}	27.59^{a}	10.59^{b}	23.58^{e}	21.47^{c}
	Kagzi Kalan	24.16^{a}	23.04^{a}	25.49^{a}	19.54^{b}	25.71^{bc}	10.08^{c}	21.55^{f}	19.89^{d}
	Hill Lemon	15.89^{c}	15.18^{e}	21.08^{e}	14.50^{f}	20.54^{f}	7.88^{f}	22.14^{f}	18.15^{e}
C. sinensis	Pusa Sharad	16.67^{b}	17.30^{dc}	23.44^{cb}	17.91^{cd}	21.29^{f}	8.06^{ef}	27.77^{dc}	23.48^{b}
	Pusa Round	15.18^{d}	16.75^{d}	22.70^{cd}	16.51^{e}	23.34^{e}	8.99^{d}	30.29^{b}	24.52^{b}
	Mosambi	16.99^{b}	17.36^{dc}	22.39^{d}	17.08^{ed}	20.59^{f}	8.52^{ed}	31.37^{a}	26.89^{a}

Values are means (n=3). Mean values in each citrus cultivar followed by different lower-case letters were significantly different at $P \leq 0.05$ by Tukey's HSD test.

Table 6. Seasonal variation in the protein content (mg g^{-1} FW) of citrus genotypes.

Species	Genotype	Month							
		Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar
C. aurantifolia	Pusa Udit	7.70^{f}	9.60^{b}	9.80^{cd}	10.40^{d}	11.02^{c}	13.60^{c}	35.76^{dc}	15.62^{d}
	Pusa Abhinav	8.10^{ef}	9.50^{b}	8.30^{e}	10.50^{d}	10.82^{c}	13.50^{c}	36.47^{bdc}	16.17^{d}
	ALC-29	9.30^{b}	9.40^{cb}	9.60^{d}	13.10^{b}	11.10^{c}	12.35^{e}	33.05^{f}	15.95^{d}
C. limon	Konkan Seedless	8.30^{ed}	10.20^{a}	10.63^{b}	14.50^{a}	13.65^{b}	14.40^{b}	37.13^{ba}	18.02^{c}
	Kagzi Kalan	10.40^{a}	10.23^{a}	13.45^{a}	12.60^{c}	16.36^{a}	16.53^{a}	34.26^{e}	18.15^{c}
	Hill Lemon	8.10^{ef}	9.50^{b}	9.80^{cd}	10.10^{d}	9.30^{d}	13.50^{c}	35.38^{ed}	19.3^{b}
C. sinensis	Pusa Sharad	8.40^{ed}	9.20^{cb}	10.20^{cb}	8.40^{f}	8.80^{e}	12.80^{ed}	37.74^{a}	19.54^{b}
	Pusa Round	8.70^{cd}	9.00^{c}	10.07^{c}	8.80^{fe}	8.90^{ed}	12.78^{ed}	36.86^{bac}	19.66^{b}
	Mosambi	8.90^{cb}	9.30^{cb}	9.88^{cd}	8.90^{e}	9.00^{ed}	13.01^{d}	37.26^{ba}	21.8^{a}

Values are means (n=3). Mean values in each citrus cultivar followed by different lower-case letters were significantly different at $P \leq 0.05$ by Tukey's HSD test.

Table 7. Seasonal variation in the level of superoxide radicals (O_2^-) (µmol g^{-1} FW) of citrus genotypes.

Species	Genotype	Month							
		Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar
C. aurantifolia	Pusa Udit	1.63^{b}	0.82^{i}	0.95^{g}	1.06^{g}	1.64^{e}	1.42^{f}	0.72^{g}	0.95^{e}
	Pusa Abhinav	1.54^{c}	1.02^{h}	1.62^{c}	1.13^{f}	1.52^{f}	1.30^{g}	0.63^{h}	0.83^{f}
	ALC-29	0.91^{h}	1.12^{g}	1.24^{f}	1.35^{e}	1.41^{g}	1.26^{g}	0.54^{i}	0.75^{g}
C. limon	Konkan Seedless	1.42^{d}	1.23^{e}	1.41^{e}	1.54^{d}	1.76^{d}	1.54^{e}	0.97^{c}	1.09^{d}
	Kagzi Kalan	1.08^{g}	1.15^{f}	1.36^{e}	1.48^{d}	1.48^{gf}	1.63^{e}	1.01^{b}	1.14^{c}
	Hill Lemon	1.73^{a}	1.84^{a}	1.92^{a}	2.04^{a}	2.23^{a}	2.54^{a}	1.12^{a}	1.32^{a}
C. sinensis	Pusa Sharad	1.32^{e}	1.46^{c}	1.64^{c}	1.87^{b}	2.04^{b}	2.26^{c}	0.84^{e}	0.98^{e}
	Pusa Round	1.64^{b}	1.71^{b}	1.82^{b}	1.93^{b}	2.15^{a}	2.37^{b}	0.78^{f}	0.87^{f}
	Mosambi	1.23^{f}	1.32^{d}	1.55^{d}	1.71^{c}	1.91^{c}	2.14^{d}	0.90^{d}	1.24^{b}

Values are means (n=3). Mean values in each citrus cultivar followed by different lower-case letters were significantly different at $P \leq 0.05$ by Tukey's HSD test.

changes occur including the carbohydrate content and respiratory enzymes. Moreover, Protein and RNA synthesis are other examples of early biochemical changes responsible for flowering in fruit trees (Marcelle, 11).

Reactive oxygen species (ROS) have been documented to have diversity of roles in the growth and development of plants besides imparting of tolerance to abiotic and biotic stresses (Das and Roychoudhary, 7). In present study, the level of O_2^- followed a gradual upward trend in most of the tested genotypes (except Pusa Udit and Pusa Abhinav limes) and attained the peak activity between December and January, which declined sharply towards the approach of February. Generally, the lower activity of O_2^- between August to January in limes and lemons (except Hill lemon) promoted the flowering (Table 7). In plants, the production of ROS is drastically increased in response to biotic and abiotic stresses. The ROS may be very damaging since they can oxidize a variety of organic molecules such as proteins, lipids and DNA (Pietta, 17) so their removal is essential. SOD detoxifies O_2^- free-radical by converting them into O_2 and H_2O_2 (Monk *et al*., 14).

The activities of active oxygen-scavenging enzymes, superoxide dismutase, catalase and peroxidase and ascorbate peroxidase have been characterized in flower buds and leaves of three different *Citrus* species for protecting plant tissue from active oxygen damage during growth, development and senescence. In leaves during the winter rest period (from August to December), enzyme activities did not change markedly. Then, leaf SOD, CAT, POD and APOX activities increased slightly in January as the buds reached towards the release the dormancy. In growing buds, the activities of the four enzymes increased strongly between dormancy and full bloom and decreased slightly post-bloom. Thus, it can be inferred that the antioxidant enzymes like POX, APOX, SOD, CAT and also a link between O_2^- production seems to be a good indicator of flowering in citrus.

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