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Analysis of various pretreatments and 2,4-D concentrations on callus induction and high efficiency of haploids regeneration in *Catharanthus roseus* (L.) G. Don.

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ABSTRACT

Androgenesis in phanerogams is a very distinct process. This technique has been proven unique breakthrough to shorten the breeding period of the cultivars for the production and fixing of agronomic traits in the homozygous state in a single generation. *Catharanthus roseus* (L.) G. Don. (Periwinkle) being an important pharmaceutical plant was selected for the investigation to study the effect of various pretreatment on callus induction in anther culture at constant concentrations of growth regulators i.e. 2, 4-D, NAA and BAP. Out of the five pretreatments chosen cold pretreatment at 8°C for 10 days was found to be the best 66.33 ± 1.74% and least was observed in centrifugation at 2000rpm for 6 hours 28.00 ± 0.57%. While 2,4-D=7mgL^{-1}, NAA=0.5mgL^{-1} and BAP=1.5mgL^{-1} were found to be suitable for maximum calli production at all concentrations of 2,4-D. Plantlets regenerated by culturing embryo like structure are all reported to be haploid.

Keywords: Androgenesis, callus, cold, homozygous, 2,4-D, pretreatment.

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INTRODUCTION

Androgenesis in phanerogams elucidates the biological mechanism behind to tipotency of a single celled microspore to develop in a double haploid plant via embryogenesis or calli formation. The process of androgenesis can be achieved by blocking the normal development of male gametophyte from its gametophytic development and forcefully diverting it to the sporophytic development. Such a diverting force is provided by means of a pretreatment to floral buds at early to late uninucleate stage of development prior to culture. Amongst the various physiological factors involved pretreatment is one of the significant factors that influence the response towards androgenesis[1,2]. A temperature shock as a pretreatment has been reported to improve the androgenetic response in many plant species[3,4]. Narkhedkaret al.[5] in preliminary studies have reported the requirement of cold shock and dark period for anther culture of *C. roseus*. Saradamani et al.[6] reported that callus induction was found to be best in 2, 4 - D. However the reports on isolated microspore culture are rather limited[7, 8, 9, 10, 11], inmajority cases, the *in vitro* response of microspores is observed within the anthers.

Catharanthus roseus (L.) G. Don. (Periwinkle) is an important pharmaceutical plant. Different parts of the periwinkle are being used traditionally for curing many diseases. Keeping this perspective, today's research aims to enhance the level of important alkaloids to satisfy the growing demand.By utilizing such a qualitative improvement practicesfor enhanced production of metabolites from dedifferentiated tissue raised by anther culture increased production of azadirachtin have been reported in *Azadirachtaindica*[12].

Ever since the commencement of modern practices of plant breeding, it aims to establish the inbreeding homozygous lines, which normally requires six consecutive inbreeding generations[13]. The importance of haploid can be judged by its utility in attaining the quick and comprehensive homozygosity amongst the offspring whose phenotype exactly reflects the genotype which reduces cultivars effort for selection of quantitative characters. Thus, in present investigation effect of various pretreatment and different level of 2,4-D concentration on callus induction was evaluated and the platform is build up for the production of homozygous lines by reporting the development of haploids in *Catharanthus roseus*.

MATERIALS AND METHOD

Donor Plant

The donor plant was selected from Botanical Garden at Department of Botany, Sant Gadge Baba

Amravati University, Amravati having GPS location N 20°56.364', E 077°48.052'. The selection of floral buds for pretreatment was done by staining the microspore in acetocarmine.

Pretreatment of Anther

Excised floral buds were having anther at early to late uninucleate stage of development were exposed to the following pretreatments.

i) Control, **ii**) Heat shock (Hst) 35^0C/3h, **iii**) Heat shock (Hst) 35^0C/6h, **iv**) Centrifugation (Cft) 2K/3h, **v**) Centrifugation (Cft) 2K/6h, **vi**) PEG 1.5% /2d, **vii**) PEG 3.0%/2d, **viii**) Mannitol 0.3M/2d, **ix**) Mannitol 0.7M/2d**, x**) Cold (Ct) 8^0C/5d, **xi**) Cold (Ct) 8^0C/10d.

At next level of optimization, anthers were cultured on 1mgL^{-1} to 10 mgL^{-1}concentrations of 2,4-D along with two combinations of BAP=0.5mgL^{-1} ($B_{1/2}$), Kinetin=1.5mgL^{-1}($K_{15/10}$) and vice versa at pre-optimized pretreatment in the present study for efficient callus production.

Explant preparation and establishment of culture

Pretreated floral buds were surface sterilized by using 0.1% $HgCl_2$and 50% ethanol. First of all, $HgCl_2$wash was given for 3 minutes followed by a single wash of distilled water for 2 minutes. Then50% ethanol was used for 2 minutes followed by two successive washes by distilled water for 4 minutes. Later, anthers were excised from buds for inoculation. Himedia's MS media, PT021, with $CaCl_2$ and Vitamins without Sucrose and Agar was employed for anther culture. Sucrose and Agar were added in the concentration of 3% and 0.8% respectively. Culture conditions were maintained at Temperature 25 ± 1°C; Relative Humidity 50–60% and complete 24 hours dark period. Control set without any pretreatment was also run.

Embryo like structures (ELS) observed on callus then subcultured on Himedia's MS media, PT100 with combinations of BAP, Kinetin and NAA and maintained at Temperature 25 ± 1°C; Relative Humidity 50–60% and 16 hours light and 8 hours dark period. Control set without any growth regulator was also run.

Ploidy analysis

Randomly thirty callus and ten leaves of the regenerated plantlets were selected for ploidy analysis by Applied Biosystems attune acoustic focusing flow cytometer.

Data Analysis

All experiments were repeated thrice and the data taken was subjected to ANOVA at 95% confidence interval through Graphpad Prism version 6.

RESULTS AND DISSCUSSION

The early to late uninucleate stage of microspore development in the anthers for pretreatment and

inoculation was deduced by acetocarmine as a nuclear stain (Figure 1 D and E). The conducted study confirmed that various pretreatments and level of 2,4-D has a significant effect ($p < 0.05$) on anther callus induction (Table 1 and Table 2). Also, it was confirmed that interaction effect of both factors was significant at 95% confidence level. The entire callus produced was rough and hard. Initially, callus was white to pale yellow at early stages of development and in prolonged culture it starts browning.

Table 1: Anther culture of *C. roseus* on MS media supplemented with growth regulators exposed to different pretreatments.

Sr. No.	Explant	Explant Number	Pretreatment	Growth regulators	Days to callus induction	% callus induced (mean ±SD)
1			Control		-	-
2	Anther	100	Hst 35°C/3h	2,4-D= 5	20	51.33±0.88
3			Hst 35°C/6h	mgL^{-1};	20	29.66±0.66
4			Cft 2K/3h	NAA= 0.5	29	29.33±1.20
5			Cft 2k/6h	mgL^{-1};	26	28.00±0.57
6			PEG 1.5%/2d	BAP= 2 mgL^{-1}	24	37.66±0.33
7			PEG 3%/2d		25	32.00±1.00
8			Mann. 0.3M/2d		26	43.66±1.33
9			Mann. 0.7M/2d		25	40.00±0.57
10			Ct 8°C/5d		20	58.66±0.66
11			Ct 8°C/10d		21	66.33±1.74

The experimental set was repeated three times. Mean within columns are significant at $P<0.05$.

Table 2: Effect of different level of 2,4-D at pre-optimized pretreatment on callus induction in Anther culture.

Sr. No.	Explant	Explant No.	Growth Regulators	Days to callus Induction	Total callus induced	% callus induction (mean ± SD)
1	Anther	50	Control	24	0.66±0.57	1.33±1.15
2			$D_1N_{1/2}B_{15/10}$	20	2.33±0.57	4.66±1.15
3			$D_1 N_{15/10} B_{1/2}$	23	3.00±0.00	6.00±0.00
4			$D_2 N_{1/2} B_{15/10}$	23	5.00±1.00	10.00±2.00
5			$D_2 N_{15/10} B_{1/2}$	21	4.00±1.00	8.00±2.00
6			$D_3 N_{1/2} B_{15/10}$	19	6.00±1.00	12.00±2.00
7			$D_3 N_{15/10} B_{1/2}$	23	7.33±0.57	14.66±1.15
8			$D_4 N_{1/2} B_{15/10}$	21	13.00±3.00	26.00±6.00
9			$D_4 N_{15/10} B_{1/2}$	18	12.66±2.08	25.33±4.16
10			$D_5 N_{1/2} B_{15/10}$	25	18.00±1.70	36.00±3.46
11			$D_5 N_{15/10} B_{1/2}$	24	19.66±0.57	39.33±1.15
12			$D_6 N_{1/2} B_{15/10}$	21	24.66±2.08	49.33±4.16
13			$D_6 N_{15/10} B_{1/2}$	22	26.00±1.00	52.00±2.00
14			$D_7 N_{1/2} B_{15/10}$	23	37.66±3.51	75.33±7.02

15	$D_7 N_{15/10}B_{1/2}$	25	35.00±1.00	70.00±2.00
16	$D_8 N_{1/2}B_{15/10}$	18	32.66±2.30	65.33±4.61
17	$D_8 N_{15/10}B_{1/2}$	24	32.66±0.57	65.33±1.15
18	$D_9 N_{1/2}B_{15/10}$	27	29.66±1.15	59.33±2.30
19	$D_9 N_{15/10}B_{1/2}$	21	30.66±1.15	61.33±2.30
20	$D_{10} N_{1/2}B_{15/10}$	21	26.66±1.52	53.33±3.05
21	$D_{10} N_{15/10}B_{1/2}$	24	26.00±0.00	52.00±0.00

The experimental set was repeated three times. Mean within columns are significant at P<0.05.

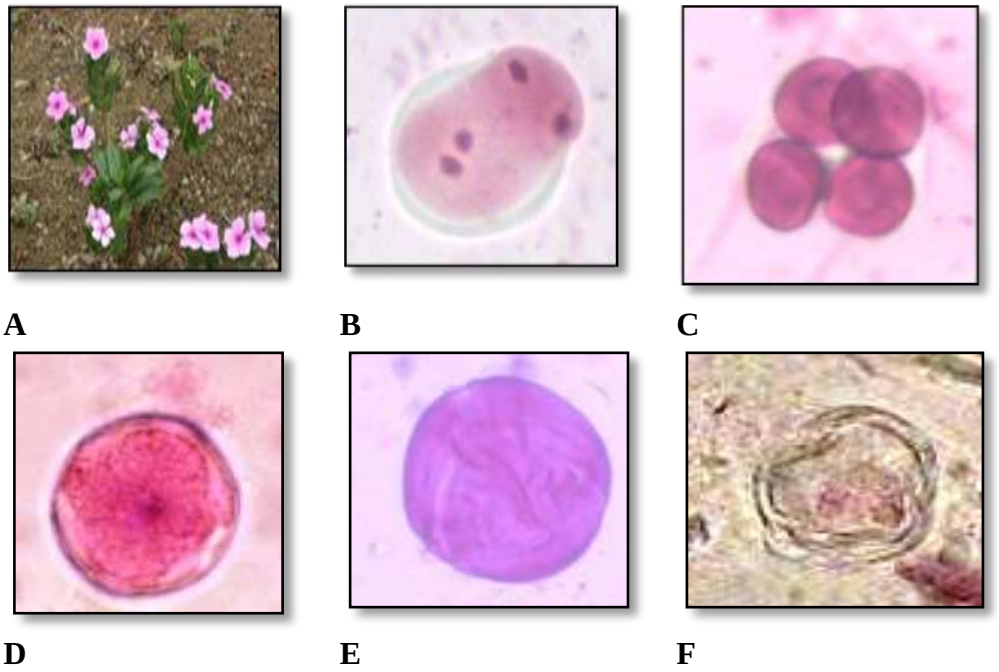
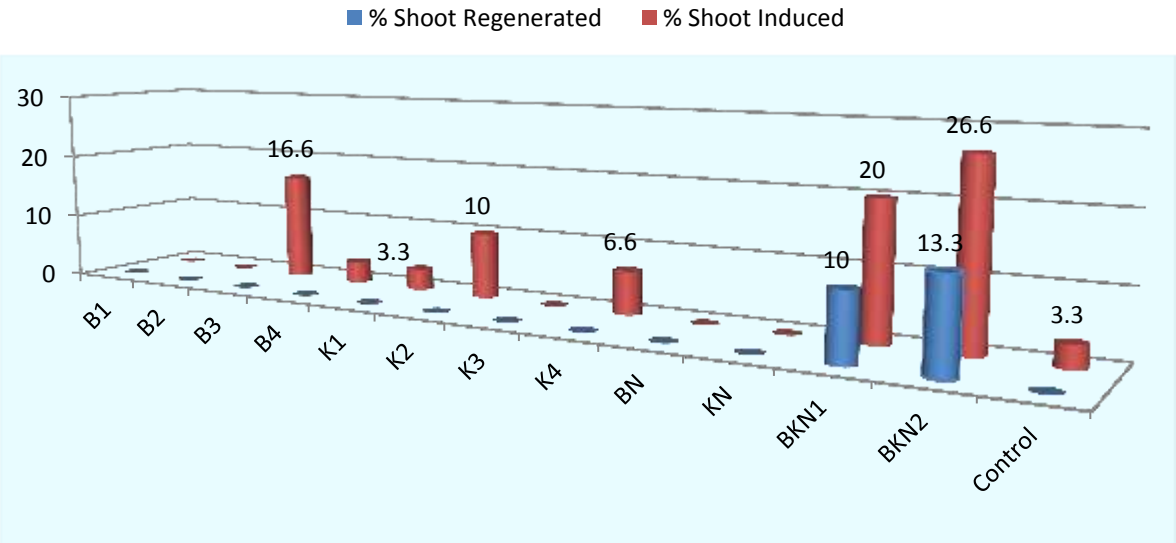


Figure1: A. Donor Plant; B-F. Pollen developmental stages (B. Pollen tetrad formation, C. Mature tetrad, D. Early uninucleate pollen, E. Late uninucleate pollen, F. Binucleate pollen)

The minimum period required for the response of anthers towards callus induction was found to be 20 days (Table 1). No response was observed in control set, this is in agreement with established fact of androgenesis that a stress must be provided to a gametophyte so as to divert it towards sporophytic development; similarly zero callus induction was reported in control set[14]. In present investigation highest rate of calli induction was reported in anthers pretreated at 8°C for 10 days i.e. 66.33 ± 1.74. The second maximal rate was again under the influence of cold treatment at 8°C but for 5 days i.e. 58.66 ± 0.66. Another pretreatment which was significant for callus induction was heat shock at 35°C for 3 hours. This signifies the importance of temperature stress in the divergence of the developmental pathway. Least response was found in centrifugation at 2000 rpm for 6 hours i.e. 28.00 ± 0.57 (Table1).Tang et al.[15] also reported that cold pretreatment (4°C/24hr) was best for callus induction in *Momordica charantia* L. followed by heat shock while centrifugation (2K rpm/12hr) has no significant effect. Likewise, Ranaweera and Pathirana[16] reported the highest rate of callus induction in floral buds incubated at 8°C for 24 hours. But

Cistue et al.[17] conclude that mannitol pretreatment was better for some varieties of Barley than cold treatment.

In the experiments on optimization of 2,4-D level, each concentration of 2,4-D is taken with two combinations of NAA and BAP (Table 2) at pre-optimized pretreatment i.e. 8°C for 10 days. 2,4-D being a potent callus inducer study was conducted at an entire admissible concentration of growth regulators i.e. 1 to 10 mgL^{-1} ($D_{1,2\ 3.....10}$). The rate of calli induction increased with increasing concentration of 2,4-D upto $7mgL^{-1}$ and later decline in the rate was observed in further increase in concentration. The best rate of callus induction was observed in 2,4-D=$7mgL^{-1}$, NAA=$0.5mgL^{-1}$ and BAP=$1.5mgL^{-1}$(Table 2). Similarly, Perera et al.[18] deduce the mutual enhancing effect of NAA and 2,4-D on pollen callus/embryo formation. According to the report on wheat by Tomar and Punia[19] an increase in the concentration of 2, 4-D in culture media produced good callus. Ranaweera and Pathirana[16] while studying on Sesame cultivar MI3 conclude that media containing 2,4-D= $10mgL^{-1}$ and $2mgL^{-1}$ each of IAA and BAP gives the maximum calli production. Confirmation of significant effect of pretreatments and 2,4-D concentration(in variables) on callus induction can be inferred by taking other parameters as a constant.



Graph 1: Callus derived ELS culture on MS media with growth regulators.

The experimental set was repeated three times. Mean within columns are not significant at P<0.05. Here, B1=BAP $0.5mgL^{-1}$; B2=BAP $1.0mgL^{-1}$; B3=BAP $1.5mgL^{-1}$; B4=BAP $2.0mgL^{-1}$; K1=KIN $0.5mgL^{-1}$; K2=KIN $1.0mgL^{-1}$; K3=KIN $1.5mgL^{-1}$; K4=KIN $2.0mgL^{-1}$, BN=BAP:NAA::1.5:0.5mgL^{-1}; KN=KIN:NAA::1.5:0.5mgL^{-1}; BKN1=BAP:KIN:NAA::0.5:1.0:0.5mgL^{-1}; BKN2=BAP:KIN:NAA:: 1.0:0.5:0.5mgL^{-1}.

Furthermore, randomly thirty callus sampled for ploidy analysis by flow cytometry showed 80% haploid callus, whereas mixoploid were 6.66% and diploids were 13.33% (Figure3 A-D). Likewise, such variation is also supported as microspore-derived calli and embryoids often show aneuploids, dihaploids, and polyploids[20].

The callus generated showed the presence of Embryo Like Structures (ELS) in more or less number irrespective of the pretreatment (Figure 2). These ELS were cultured on twelve combinations of growth regulators along with one control set showed a limited response towards plant regeneration and the establishment of stable culture. Out of thirteen sets, only eight experimental sets showed shoot induction by forming bifurcation and green coloration at an apical position above the media. But only two combinations i.e. BAP:KIN:NAA::0.5:1.0:0.5mgL^{-1} and BAP:KIN:NAA::1.0:0.5:0.5mgL^{-1} found to produce plantlets (Graph 1, Figure 4). These regenerated plantlets could attain a maximum of 3.0±0.2cm shoot length. Establishment of prolonged culture was not successful in the experiments performed. In case of callus induction, Kim et al.[21] obtained callus in media supplemented with 1mgL^{-1} NAA and 0.5 mgL^{-1} Kinetin. Singh et al.[22] reported maximum calli in MS media with BAP and NAA at 1mgL^{-1} and shoot proliferation at BAP 1.5mgL^{-1} and NAA 1.0mgL^{-1} for *Catharanthus roseus*. Multiple shoot induction was reported by Mehta et al.[23] in MS medium supplemented with 0.5 mg/l BAP ± 1mg/l NAA.

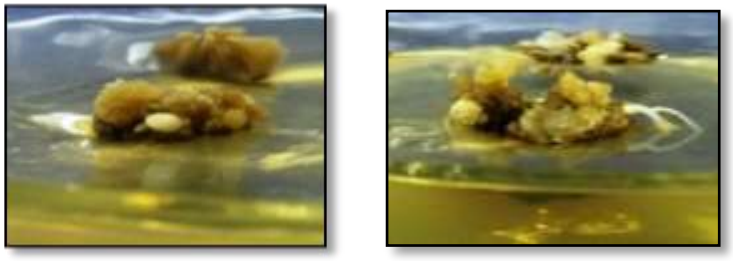


Figure 2: Callus induction in periwinkle showing ELS in anther culture of cold pretreated buds at 8°C for 10 days.

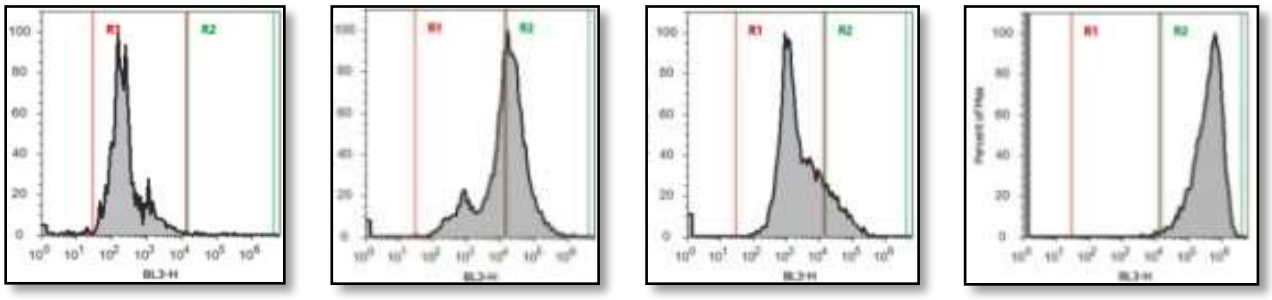
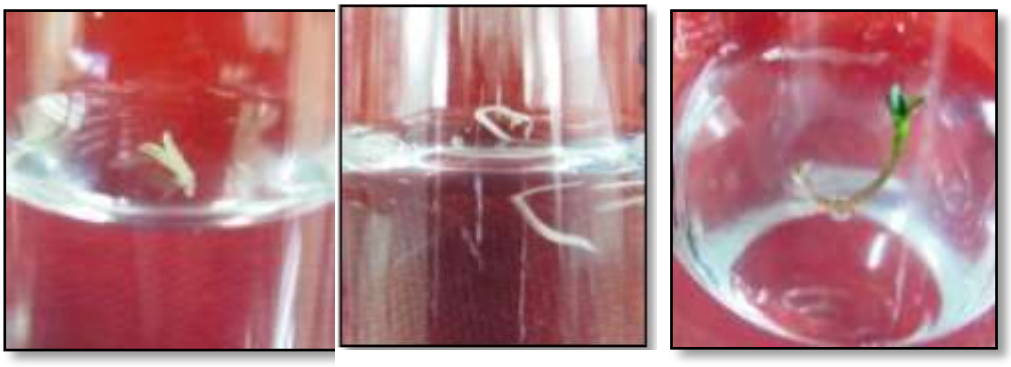


Figure 3: Flow cytometry analysis. A. Diploid standard, B-D. Variation in ploidy status of callus (B. Haploid, C. Mixoploid and D. Diploid)



A B C

Figure 4: Successive stages of plant regeneration from ELS in MS media with BAP 1.0mgL^{-1}, KIN 0.5mgL^{-1} and NAA 0.5mgL^{-1}.

Moreover, indiscriminately all ten leaflets sampled and analyzed for ploidy status showed haploidy (Figure 5 B). However, Kim et al.[21] reported the high frequency plant regeneration from anther derived cell suspension culture via somatic embryogenesis in *Catharanthus roseus* but plant regenerated were all diploid.

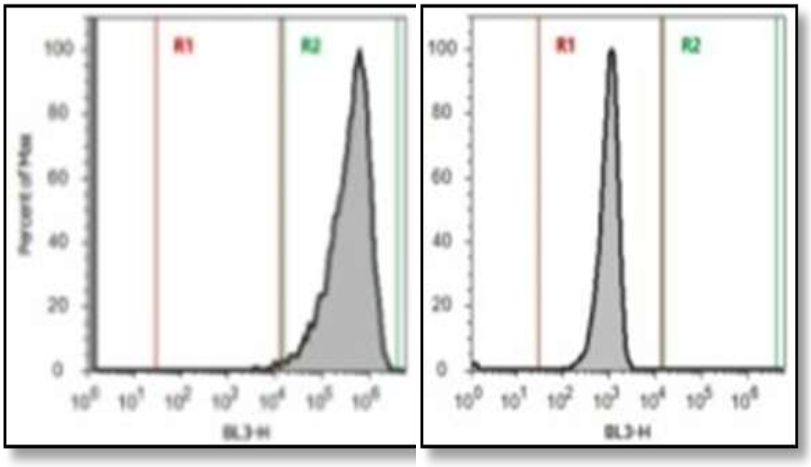


Figure 5: Flow cytometry analysis. A. Diploid standard, B. Haploid (Leaves of regenerated plant)

CONCLUSION

From the findings, it is concluded that pretreatment and 2,4-D concentration has a significant effect on the calli induction. Wherein, cold pretreatment at 8°C for 10 days is most significant and least effective noted was centrifugation at 2000 rpm for 6 hours. Whereas, 2,4-D at 7mgL^{-1} concentration was best for callus induction. Haploid induction efficiency was higher than spontaneous diploidization in the experiment carried out. Besides, a protocol needs to be optimized for the establishment of stable culture and dihaploid plant regeneration.

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