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ORIGINAL RESEARCH ARTICLE

Influence of brassinosteroids on sex allocation in walnut (*Juglans regia* L.) flowers

Hakan Engin, Zeliha Gökbayrak*

Department of Horticulture, Faculty of Agriculture, Çanakkale Onsekiz Mart University, 17100 Çanakkale, Türkiye. E-mail: zgokbayrak@comu.edu.tr

ABSTRACT

The effect of foliar treatment with brassinosteroid (BR) on gender distribution in flowers of walnut (*Juglans regia* L. cv. Chandler) was investigated. Grafted walnut saplings ('Chandler') on the wild walnut (*Juglans regia* L.) rootstock were planted into 70-liter pots with a soil: peat: perlite medium and grown in pots between 2016–2020. BRs (24-epibrassinolide; EBR and 22(S), 23(S)-homobrassinolide; HBR) were applied at a concentration of 1 mg L^{-1} for four consecutive years at the time of flower differentiation. The experimental design was completely randomized with three replicates. The results show that BR applications could alter the sexual distribution of the walnut's flower. BRs application significantly increased the number of total flowers and female flowers per tree. The number of female flowers was also increased by the season. The highest number of female flowers (20.9) was observed in the trees in 2020 and the application of 1 mg L^{-1} of HBR. It was determined that the annual growth of the plant and the increase in the number of females and total flowers were positively related. The effect of BRs indicated that the response was BR-type specific.

Keywords: Catkin; Chandler; Female Flower; Homobrassinolide; *Juglans regia* L.

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1. Introduction

Reproductive meristems of a fruit tree go through many developmental processes with significant coordination between the environmental stimuli and inherent characteristics. Having it complete with a transition from a vegetative phase to a fruitful one with a high success rate leads to flowering and ensures a good yield if all goes well later. Aside from a complete dependence on weather conditions, flower formation also is regulated by internal stimuli such as phytohormones. However, the coordinating signals for floral organ development have been long away from total elucidation[1].

Although once believed that plants do not change the types of flowers[2] during their lives, it is now well documented that age, dry soil, high light intensity, etc., could cause a gender difference[3,4]. It's been suggested that exogenous phytohormone, or plant growth regulator (PGR) applications might have an influence on flower gender[5,6] and help regulate sexual expression in flowers. Reports of PGRs, mainly auxins, gibberellins, and cytokinins, influencing floral differentiation, causing a shift towards the opposite gender or increasing the number of one gender against the other have been presented[4,7–11]. Gao *et al*.[12] found that exogenous application of PGRs could shift male flower primordium to a vegetative one. The general conception gathered from these studies is that the determination of flower sex in response to PGRs is species-specific.

Brassinosteroids are relatively new type of signaling molecules, whose effect on floral development is under the spotlight. Brassinosteroids exhibit organic similarities with animal steroids which show attributes of sex determination in animals[13]. Its significant role in other development has been shown[1]. Papadopoulou *et al*.[14] reported diverse effects of brassinolide on staminate and pistillate initiation. Engin and Gökbayrak[15] found no clear effects on the types of flowers in pomegranate. Interfering with brassinosteroid synthesis was shown to affect meristems turning into inflorescences in *Setaria viridis*[16] and in *Zea mays*[17,18]. Bisexual flowers were formed in mutated plants of *Arabidopsis*, *Lycopersicon esculentum* Mill., *Pisum sativum*, and *Oryza sativa* for BR synthesis[19]. Compared to the well-studied phytohormones in terms of the floral response of plants, there are still much needed studies on finding out the effects of the brassinosteroids on flower differentiation and formation.

Juglans regia L., aka Persian or English walnut, is a significant member of the Juglandaceae family. It has edible nuts and is cultivated widely in the Northern Hemisphere. It is a monoecious species with male and female flowers borne on the same tree but placed separately from each other[20]. Walnut, carrying two types of flowers, is a good species to study and observe the effects of any kind of plant hormones exogenously applied. The species forms its staminate and pistillate flowers at different positions in the tree, enabling easy count and process. Catkins, the group of male flowers, are positioned laterally as opposed to the female flowers appearing terminally[20].

The objective of the present study was to investigate the effects of epibrassinolide and homobrassinolide, two brassinosteroid compounds prominent with their low-dose effects, on the staminate and pistillate flower allocation in the "Chandler" walnut cultivar.

2. Material and method

2.1 Plant material

The research was carried out at Çanakkale Onsekiz Mart University, Faculty of Agriculture, Department of Horticulture, between 2016 and 2020. The "Chandler" walnut was grafted on wild walnut (*Juglans regia* L.) rootstock in a private nursery and developed in the nursery until February 2016. They were later planted in 70-liter pots filled with a mixture (2:1:1) of soil, peat, and perlite, and grown for the duration of the experiment for 4 years (**Figure 1(a)**). Meteorological data for the duration of the study (2016–2020) were provided in **Table 1**.

Figure 1. Male and female flowers of *Juglans regia* L. cv. Chandler. **(a)** grafted saplings in 70-liter pots, **(b)** male flowers, **(c)** an individual female flower, **(d)** double female flowers developed, and **(e)** triple female flowers developed.

2.2 Brassinosteroid applications

Two different types of brassinosteroids (BRs), 24-epibrassinolide (EBR) and 22(S), 23(S)-homobrassinolide (HBR) were used. Both BRs were applied at a concentration of 1 mg L^{-1}. Solutions were prepared by dissolving in small volumes of 96% ethyl alcohol, and a final volume was made by adding pure water. Three drops of polysorbate 20 were added to the solution. Only pure water with polysorbate 20 was applied to the saplings in the control group. BRs applications were sprayed using a hand sprayer onto the stem, branches, and side branches of the saplings until they were completely wet (about 100–200 mL). Both BRs were applied twice at one and two months after fruit set (time of flower differentiation; Gao *et al*.[21]). BRs applications

Table 1. Meteorological data between 2016 and 2020 for Çanakkale-Türkiye region

Maximum temperature (°C)												
Year/month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
2016	18.1	20.9	20.5	27.6	25.9	36.7	35.1	35.5	32	28.1	24.2	15.7
2017	14.8	16.7	21.9	25.2	38.9	36.2	39	39.1	34.1	24.5	22.1	20
2018	14.9	15.7	19.6	29.2	28.6	31.8	33.9	33.8	34.9	24	22.6	16.9
2019	15.9	15.9	20.8	24.1	33.3	36.2	36.8	36.7	33.6	30.6	25.4	19.7
2020	16.5	18	20.9	24	32.1	35.4	36.7	36.1	35	31.8	20.8	18.2
Minimum temperature (°C)												
Year/month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
2016	–6.5	–1.3	0.5	6.6	9.2	13.2	19.1	19.4	11.5	6	0.9	–2.5
2017	–6.4	–2.5	1	4.5	9.1	15.3	18	16.7	12.2	6.4	2.3	0
2018	0.4	1.2	0.4	7.1	10.1	13.5	19.3	19.9	12.9	5.7	0.7	–0.8
2019	–2.2	–2.5	0.9	3.8	8.1	14.9	16.7	18	12.2	10.5	7.3	2
2020	–0.6	–1.5	1.2	3.9	8.8	13.1	17	18.8	15.7	8.1	3.6	1.9
Average temperature (°C)												
Year/month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
2016	7	10.9	11.2	15.7	18.2	24.5	26.9	27	22.5	17.1	12.5	5
2017	3.9	7.9	10.6	13.3	18.7	23.9	26.6	26.7	22.2	15.7	12.5	11.1
2018	7.8	8.8	11.9	15.6	19.6	23.2	26.5	27.1	22.3	17.4	13.1	7.5
2019	7.7	7.2	10.8	13.4	19.6	25.8	26.7	27.5	23.4	19.4	17.5	11.2
2020	7.3	9.7	11.7	12.3	18.2	22.6	27	27.1	24.7	19.3	12.7	11.5
Mean relative humidity (%)												
Year/month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
2016	72.9	77.9	74.4	67.9	70.1	62.3	55.4	59	60.1	65.9	70.5	69.1
2017	76.8	74.9	73.5	64.4	65.3	64.1	56	53.6	58	67	75.1	73.2
2018	76.5	80.6	77.3	67.5	70.1	63.9	59.6	56.5	63.5	72.5	75.8	78
2019	76.1	75.6	69.2	69.1	64.7	58.7	52	52.8	54.2	67.5	71.6	71.5
2020	67.6	69.3	68.6	68.2	68.9	74	55.3	54.2	59.6	77.5	79.4	87.3
Total precipitation (mm)												
Year/month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
2016	110.2	88.4	53.6	15	26.8	39.9			1.8	8.6	210.3	16.3
2017	155.6	56.8	22.1	14.9	19.3	36.7	17.2		11.7	58.3	117.5	146.5
2018	88.4	133.5	57.8	16.6	32.5	18.8	16.7		72.7	33	92.8	97.3
2019	93.2	68.4	64.5	86.6	4.5	56.8	19.6	10.5	1	34.8	18.8	47.2
2020	57.2	48	24.3	55.7	54.6	38.8	0.1	3.2	9.5	51.3	0.7	113.8

were repeated in the same periods for 4 consecutive years, starting from 2016.

2.3 Determination of flower sex distribution

The scoring of male, female, the ratio of male to female (**Figure 1(b)–(e)**) and a total number of flowers were carried out in the springs, starting from 2017 to 2020.

2.4 Statistical analysis

The research was carried out according to the completely randomized design with 3 replications. 4 saplings were used in each replication. The data obtained at the end of the study were evaluated at the $p < 0.05$ level performing two-way (year and application) analysis of variance of the Minitab 17 statistical package program. Comparison for the means was done by using Tukey's *t*-test. A second analysis, ANOM (analysis of means) was also performed at alpha level 0.05 on R statistical package program (version 4.0.2; 2020–06–22)[22]. In *R* analyses, the differences between the applications are made according to the general mean, indicating that the groups that are out of the upper and lower limits are statistically more important than the other groups.

Since both analyses revealed the same type of effects of the factors on the parameters, ANOM charts were used to illustrate the results because of their easiness of understanding. Both upper and lower decision lines (UDL and LDL), as in confidence interval computation, were calculated.

3. Results

Descriptive statistics and results from the comparison test following ANOVA were presented in **Table 2**. It showed that the number of catkins per tree and the ratio of catkins flowers to pistillate were independently affected by the application seasons and the treatments. Number for the pistillate and total flowers were under the effect of interaction.

Table 2. Descriptive statistics of the parameters depending on the seasons and BR treatments (means ± SEM) and Tukey's *t*-test following ANOVA

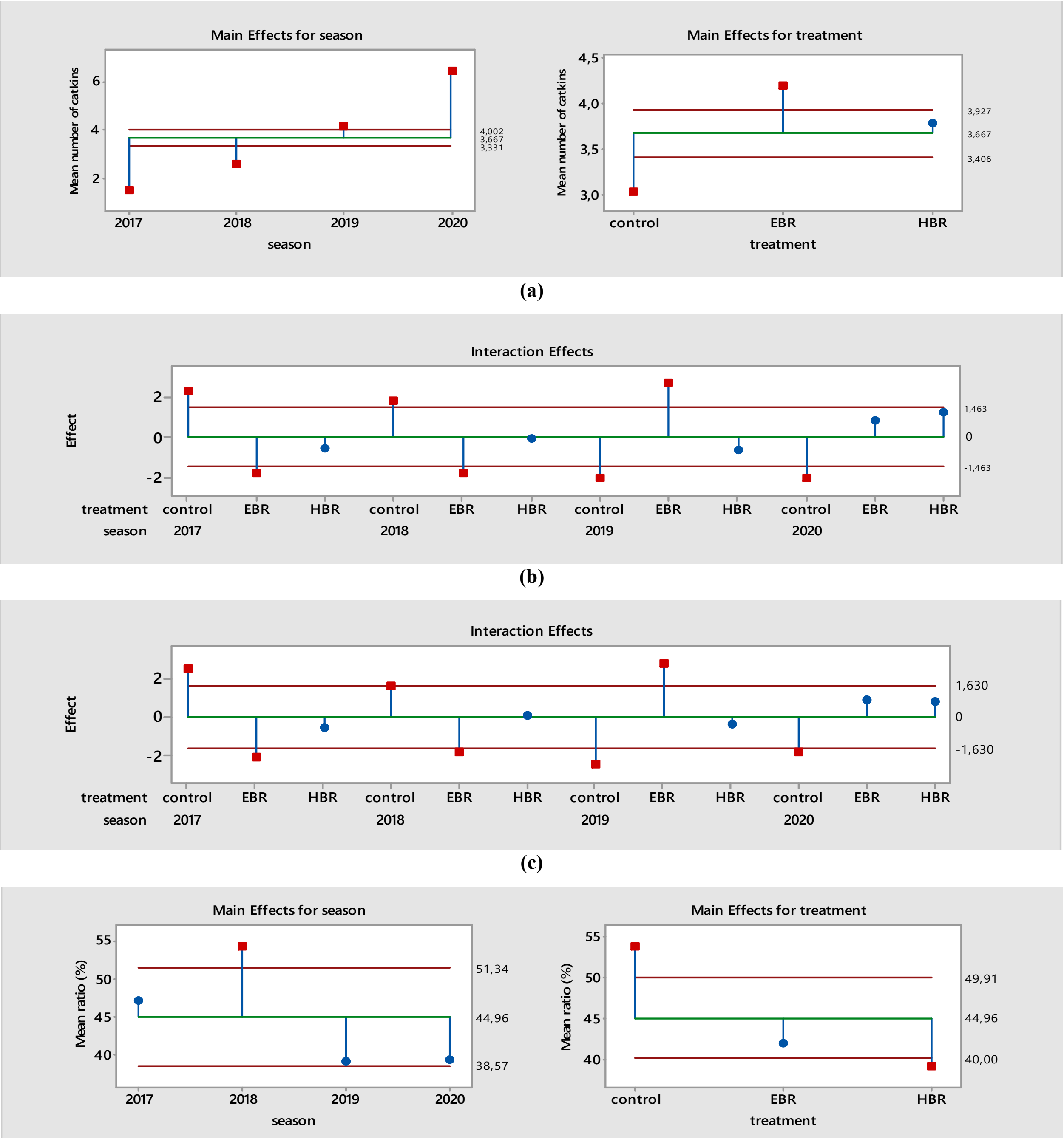
Catkins (number per tree)				
	EBR	**HBR**	**Control**	**Mean**
2017	1.78 ± 0.15	1.67 ± 0.17	1.11 ± 0.11	1.52 ± 010 d
2018	3.11 ± 0.20	2.78 ± 0.22	1.78 ± 0.28	2.56 ± 0.17 c
2019	4.78 ± 0.22	4.56 ± 0.41	3.11 ± 0.26	4.15 ± 0.23 b
2020	7.11 ± 0.35	6.11 ± 0.26	6.11 ± 0.39	6.44 ± 0.21 a
Mean	11.86 ± 1.27 A*	10.78 ± 1.12 B	5.94 ± 0.69 C	
Female flowers (number per tree)				
	EBR	**HBR**	**Control**	**Mean**
2017	4.22 ± 0.40 Ca	4.33 ± 0.37 Ca	2.33 ± 0.33 Ca	3.63 ± 0.27
2018	5.44 ± 0.29 Ca	6.11 ± 0.48 Ca	3.11 ± 0.20 BCa	4.89 ± 0.32
2019	16.89 ± 0.98 Ba	12.44 ± 0.84 Bb	6.22 ± 0.36 Bc	11.85 ± 0.96
2020	20.89 ± 1.06 Aa	20.22 ± 1.26 Aa	12.11 ± 0.86 Ab	17.74 ± 0.98
Mean	11.86 ± 1.27	10.78 ± 1.12	5.94 ± 0.69	
Total flowers (number per tree)				
	EBR	**HBR**	**Control**	**Mean**
2017	6.00 ± 0.47 Ad	6.00 ± 0.44 Ad	3.44 ± 0.38 Bd	5.15 ± 0.34
2018	8.56 ± 0.29 Ac	8.89 ± 0.54 Ac	4.89 ± 0.45 Bc	7.44 ± 0.43
2019	21.67 ± 0.99 Ab	17.00 ± 1.07 Bb	9.33 ± 0.53 Cb	16.00 ± 1.11
2020	28.00 ± 0.96 Aa	26.33 ± 1.41 Ba	18.22 ± 1.00 Ca	24.9 ± 1.05
Mean	16.06 ± 1.58	14.56 ± 1.41	8.97 ± 1.02	
Ratio of catkins to females (%)				
	EBR	**HBR**	**Control**	**Mean**
2017	44.26 ± 5.02	40.56 ± 5.63	56.48 ± 9.19	47.10 ± 4.04 ab
2018	59.21 ± 6.17	48.07 ± 5.01	55.56 ± 6.05	54.28 ± 3.33 a
2019	29.24 ± 2.46	37.30 ± 3.57	50.69 ± 4.60	39.07 ± 2.67 b
2020	34.97 ± 2.65	30.73 ± 1.48	52.42 ± 4.55	39.37 ± 2.54 b
Mean	41.92 ± 2.83 B	39.16 ± 2.28 B	53.79 ± 3.08 A	

*Tukey's *t*-test comparison at 95% confidence level, capital letters indicate effects of treatments in a year, and small letters indicate effects of seasons in a treatment ($p < 0.001$).

ANOM chart for the number of catkins (**Figure 2(a)**) displayed that the interaction effects were not significant and the significance of the main effects of the seasons and the BR treatments were observed. The overall mean for the main effects of the season was 3.667 and the mean values in the first two and latest two growing seasons were well below and up than the overall mean, indicating that seasonal differences caused significant differences.

The number of staminate flowers continued to increase throughout the experiment. The effects of the BRs, on the other hand, were easily distinguishable between the control group and the BR-applied group. It was observed that while EBR produced comparably more catkins than HBR, implicating BRs might be involved in producing more male flowers. It is possible to speculate that EBR had a more masculinizing effect compared to HBR.

The number of female flowers produced was influenced by the season and treatment combination (**Figure 2(b)**). In the first two years, similar effects were observed and HBR produced more female flowers compared to EBR, but both stayed on the lower side of the overall mean. The control plants had noticeably more female flowers. However, later in the growing seasons, different effects of the type of BRs could be noticed. In 2019, the third year of the experiment, EBR increased the number of pistillate flowers, but the increase was lower in 2020. The response to HBR was, on the other hand, well within the decision limits, while it still produced more females compared to control plants.



(a)

(b)

(c)

(d)

Figure 2. ANOM decision charts for **(a)** number of catkins per tree, **(b)** number of female flowers per tree, **(c)** number of total flowers per tree, and **(d)** ratio of catkins to female flowers per tree, in "Chandler" walnut after treatments with brassinosteroids over 4 growing seasons.

As for the female flowers, the total number of flowers was also under the influence of the interaction between the seasons and BRs (**Figure 2(c)**). Control plants had the highest number of flowers in the first two seasons, with higher mean values than the overall mean. BR treatments were on the below side, with EBR acting significantly differently than HBR. However, in the 3rd season (2019), the total number of flowers considerably declined, and EBR caused an increase in the opposite direction. The same type of tendency continued in the last season of the experiment and both BRs had similar but higher mean values than control plants. It was observed that EBR was more successful in increasing flower numbers.

The ratio catkins to female flowers are shown in **Figure 2(d)**. It indicated that there were main effects at play. As the seasons progressed starting from 2017, the ratio went up, meaning that there were more catkins than females. But in 2019 and

2020, the ratio was lower and similar, indicating that the number of female flowers increased as opposed to the male flowers. The response to the BR treatments showed that BRs caused a decrease in the catkin numbers and that HBR was more effective than EBR.

4. Discussion

24-epibrassinolide and 22(S), 23(S)-homobrassinolide were applied to the trees of "Chandler" walnut cultivar to find out the possible effects of brassinosteroids on flower formation in terms of gender distribution. It was stated that flower production along with the ratio of male to female flowers in Juglandaceae changes with various factors[23,24].

The results of this study indicated that seasonal differences played a role in the gender formation in the walnut trees. Whether a flower will be male, or female is determined by the summer previous to fruiting when the flower buds are going through their initial development[25]. During this period, the new developing buds on walnut trees are sensitive to any type of climatologic conditions that affect the tree's growth. Big differences between day and night temperature showed a diminishing impact on flower bud formation[26]. Stromberg and Patten[3] reported yearly variations in the female and male flowers and stress-related increases in the maleness in *J. major*. The floral buds are found to highly responsive to be high temperatures (above 30 °C) at the beginning of their differentiation[27]. In this study, the ratio of catkins to females in 2018 was higher than that in 2017, 2019, and 2020. This might have been due to high maximum temperature in 2017 (38.9 °C in June and 39 °C in August and September, see **Table 1**) during flower differentiation previous year. The average temperature during flower differentiation in 2018 and 2019 showed a parallelism with an increase in the number of total flowers. It was speculated by Yuan *et al.*[28] that environmental signals may be prompted by brassinosteroid signaling to coordinate floral transition at a certain time at the gene level. The onset of differentiation could be impeded by the earlier cool weather, as observed in 2017 and 2019. However, variations in the time of onset in this study are not attributed to heat accumulation between full bloom and the onset of floral initiation.

The main effects of the type of BRs were also observed on the catkin numbers and the ratio of male to female flowers. EBR-treated plants had more male flowers and HBR-treated ones contained more female flowers. Their involvement in sex formation in flowers is not clear yet, but Ishii *et al.*[29] reported in their study with BR-deficient mutants that brassinosteroids might be involved in flower formation in *Fragaria vesca*. Epibrassinolide applied to *C. pepo* plants produced more flowers, with a shift towards feminization[30]. Papadopoulou *et al.*[14] indicated a diminishing effect on maleness, while Manzano *et al.*[31] reported a possible positive regulation of femaleness in the cucumber. Mutum *et al.*[32] also reported similar effects of brassinolide on papaya. These varying results particularly indicate that the response of floral buds is species- and type-specific.

5. Conclusion

Brassinosteroid applications affected the number of female and male flowers in the "Chandler" walnut cultivar. Applicability of the results obtained from plants in pots might be challenging compared to orchard conditions. However, as more empirical information is gathered on the effects of brassinosteroids on shifting one flower type to another, it might enable walnut producers to include spraying the trees with a certain type of these phytohormones, which lead to more yield and hence, more income.

Author contributions

Conceptualization, HE and ZG; methodology, HE and ZG; validation, HE; formal analysis, ZG; investigation, HE and ZG; resources, HE and ZG; data curation, HE; writing—original draft preparation, HE; writing—review and editing, ZG; supervision, ZG. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

Authors declare that they have no competing interests.

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