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A hybridization technique for orphan legumes: development of an artificial interspecific pollination protocol for *Crotalaria spp*.

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ABSTRACT

Crotalaria brevidens: and *C. ochroleuca* commonly grown as vegetables have been extensively studied. However, information on breeding techniques is scanty. This study aimed to develop an artificial interspecific pollination protocol for two genotypes of *Crotalaria* with contrasting leaf and pod morphological features. The two are landraces that were previously collected from two counties in Kenya. The experimental design was a completely randomized design (CRD) in a greenhouse with *C. ochroleuca* as the female parent and *C. brevidens* as the male parent. Six-day-old flower buds of the female parent were emasculated. Pollen from a freshly opened flower was rubbed over the stigma of the emasculated flower. The pollinated stigma was inserted back into the keel petal and covered by the wing and standard petal. Data on success rate, pod, and seed production were subjected to analysis of variance. The developed artificial pollination method showed 75% success rate in the interspecies cross of *C. ochroleuca* and *C. brevidens*. Time of crossing did not significantly influence the success rate, pod, and seed production. The study demonstrated artificial interspecific pollination of *Crotalaria* by keel petal incision rubbing method. This protocol lays the foundation for genetic studies and improvement of *Crotalaria* spp.

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Crotalaria; artificial pollination; keel petal incision; rubbing method; pollination protocol

1. Introduction

Crotalaria species are annual herbs in the family Fabaceae and sub-family Papilionoideae (Etcheverry, Alemán, and Fleming 2008). They are often considered to be weeds that colonize roadsides, open fields, and human-interfered areas (Devecchi, Pirani, and Melo-de-Pinna 2014). The genus consists of a number of important indigenous crops, whose young shoots and leaves are consumed as green vegetables (Gido, Bett, and Bokelmann 2016). The two edible species are *C. ochroleuca* and *C. brevidens* (Abukutsa-

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Onyango 2007). *C. ochroleuca* is found in damp grassland, along riverbanks, roadsides, and fields. It grows in areas with adequate sunshine at 300–2000 m above the sea level, whereas *C. brevidens* grows in open, wooded grassland and sometimes in seasonal swamps at 500–2700 m above sea level (Wang, Sipes, and Schmitt 2002).

The genus *Crotalaria* has diverse uses, such as making cordage, canvas, fishing nets, and cigarette papers. It consists of more than 600 species of herbs and shrubs that are wild or domesticated. *Crotalaria micans* is used as edible vegetable in North eastern India (Subramaniam and Pandey 2014), whereas *C. ochroleuca* is consumed and used as a green manure crop in Uganda (Fischler, Wortmann, and Feil 1999). *C. juncea* (sunn hemp) is native to India and is largely cultivated in India, Brazil, Bangladesh, Pakistan, China, and Korea. It is suitable for a variety of uses in rural households and industry (Bhandari et al. 2016). In East Africa, there are only two documented edible species of *Crotalaria*, i.e., *C. ochroleuca* and *C. brevidens* Benth (Nduhiu 2017). These two species are commonly characterized as "slender leaf." Slender leaf is among the African leafy vegetables that are grown and consumed in Kenya. The young leaves and shoots are used as a cooked vegetable. The vegetable provides 100% of the dietary requirement for vitamin A, vitamin C, iron, calcium, and 40% of proteins from the consumption of 100 g of fresh weight (Abukutsa-Onyango 2007).

C. ochroleuca is characterized by its pale-yellow flower color, whereas *C. brevidens* has bright-yellow flower color. Its leaves are bright green and the plant can grow to a height of 250 cm. The pods of *C. ochroleuca* are bigger or wider in diameter than those of *C. brevidens* (Abukutsa-Onyango 2007). *C. brevidens* has bluish-green leaves and grows to a height of 210 cm with small slender pods. The seeds of *C. ochroleuca* are pale yellow to brown, whereas seeds of *C. brevidens* are pale yellow, turning orange to dark red (Al-Snafi 2016). The key distinguishing features between *C. ochroleuca* and *C. brevidens* are the pod size and taste. *C. ochroleuca* has a mild taste with big wide pods, whereas *C. brevidens* is bitter with small slender pods (Nduhiu 2017).

The genus *Crotalaria* inflorescence has a terminal raceme of up to 50 cm long. The flowers are bisexual, hairy to glabrous with five stamens that have long anthers and five stamens with small rounded anthers. The wing petals are as long as the keel. The keel has a long beak of up to 2.5 cm long, with 10 stamens joined in an open sheath at the base, with a curved style and small stigma (Tripathi et al. 2013). *C. brevidens and C. ochroleuca* are self-compatible; however, some species of *Crotalaria*, such as *C. juncea*, can be cross-pollinated by insects, such as carpenter bees belonging to the genus *Xylocopa* (Etcheverry, Protomastro, and Westerkamp 2003). Its pollen is masked and presented only when authentic pollinators, such as large bees, which are strong enough to weaken the keel, expose the stigma and push out a mass of pollen grains (Yaradua 2018).

Artificial hybridization provides an opportunity to increase genetic variability. In particular, interspecific crosses allow for the introgression of important alleles from wild species, e.g., alleles for resistance or tolerance to abiotic and biotic stresses; however, the presence of strong pre- and post-fertilization barriers is a major constraint in hybridization programs. Application of pollen biotechnology (such as *in vitro* fertilization, mentor pollen, and embryo rescue) to conventional breeding programs not only decreases the time and cost involved but also greatly increases the efficacy of the conventional breeding methods.

The use of an appropriate bud for crossing, provision of viable pollen, and suitable equipment has been shown to determine the success rate of crossing (Ray et al. 2003). Pollination of the self-pollinated *Crotalaria* species is ensured by dehiscence of the anthers before anthesis (Jacobi, Ramalho, and Silva 2005). The floral morphology and reproductive biology of Papilionoideae plants in the family Fabaceae are well studied. However, there is only limited information available about the pollination biology and interspecies pollination of *C. brevidens* and *C. ochroleuca*. Therefore, this study aimed to develop an artificial interspecies pollination protocol for *C. brevidens* and *C. ochroleuca.*

2. Materials and methods

2.1. *Study site and description of experimental plants*

This study was conducted in a screened greenhouse at the University of Embu Farm (0° 35′ 25” S, 37° E 25′ 31”). The two distinct genotypes, *C. brevidens,* and *C. ochroleuca* landraces were collected from Homa Bay and Kakamega counties in Kenya. *C. ochroleuca* was used as the female parent, whereas *C. brevidens* was the male parent. *C. brevidens* is an early-flowering cultivar designated as Fkk 0039 landrace. It has an intermediate height of 1.3 m to 1.5 m, pod diameter of 0.82 cm to 1.0 cm, which is described as a slender or small pod, and leaf length of 5.0 cm to 6.0 cm with lanceolate leaflet shape. *C. ochroleuca* is a late-flowering cultivar designated as Fhb 0211. It has intermediate height of 1.37 m to 1.5 m, pod diameter of 1.0 cm to 1.8 cm, referred to as big pod, and leaf length of 4.5 cm to 6.5 cm with elliptical leaflet shape (Figure 1).

2.2. *Experimental layout and management*

The experiment was set up in July 2019 in a completely randomized design (CRD) with six treatments replicated thrice. The experimental units comprised of 20.0 cm diameter plastic containers filled with 3 kg medium substrate made up of farm soil, farmyard manure, and sand in a ratio of 3:2:1. Three *Crotalaria* seeds were planted in the medium substrate and later thinned to one. Eighteen containers were used per treatment. A total of 54 seeds were planted per landrace before thinning.

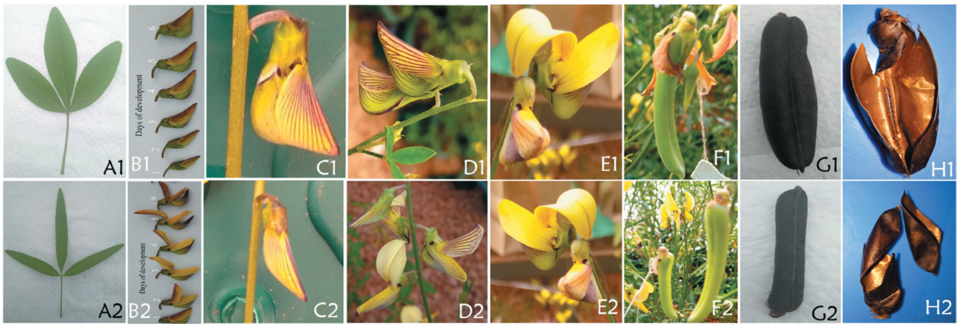

Figure 1. Morphological features and floral development in *Crotalaria* genotypes used for interspecies pollination. A- leaf shape (A1 – lanceolate, A2 – elliptical), B – flower bud stages, C – flower bud appropriate for emasculation, D – open flower bud, E – outer *Crotalaria* flower structure, F – pod development after artificial pollination, G – pod size (G1-big pod, G2 – slender pod), H – dry aborted pod with no seed. Suffix 1 on photo labels denotes *C. ochroleuca*, suffix 2 on the photo labels represents *C. brevidens*

2.3. *Emasculation*

Six-day-old flower buds, which were about to open, were selected (Figure 1) and mechanically emasculated using a pair of forceps. The bud was gently grasped to prevent any kind of stress or injury. A small, finely pointed pair of forceps was used to make a cut at the bottom of the bud to allow access to the anthers and the stigma. The flower was held on the thumb and supported by the index and the middle fingers. Using a sharp tip of a scalpel, an incision was made along the center of the keel (Figure 2). This exposed the stamens and the pistil of the flower, granting easy access to the male and female parts of the flower. All 10 anthers, 5 long and 5 short ones, were carefully removed one by one, without damaging the stigma. Between crosses, the pair of forceps was sterilized by dipping in 70% ethanol to avoid cross contamination.

2.4. *Pollination*

Pollination was carried out immediately after emasculation (Figure 2). Freshly opened flowers were obtained from desirable males and used immediately. To expose the anther sac, the inner petals of an open flower were slipped downwards by use of a pair of forceps to expose the mass of pollen grains. Thick pollen emerged after the wings were pressed downwards. The pollen grains were gently rubbed on the stigma of the female plant using forceps to ensure that the pollen sticks on the stigma. The stigma was returned to the keel and closed with the wing and the standard petals immediately after pollination to avoid desiccation. To investigate the effect of pollination time on pollen viability and hybridization success, pollination was done in the morning (08:00 hours) and in the evening (17:00 hours) and the data for the two events were recorded separately.

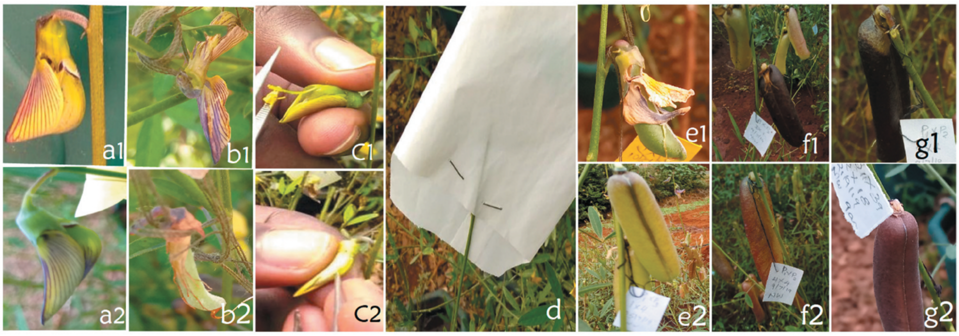

Figure 2. Emasculation and artificial pollination of two *Crotalaria* genotypes. (a) – flower bud chosen for emasculation, (b) – abortion, (c) – emasculation and pollination process, (d) – protection of pollinated flower bud, e – pod development after artificial pollination, (f) – mature pod after 30 days, g – mature pod ready for harvesting. Suffix 1 labels represent *C. ochroleuca*, suffix 2 labels represent *C. brevidens*.

2.5. *Tagging and protection of flower buds*

The emasculated, artificially pollinated flowers were tagged and labeled for identification. The tags were attached to the flower pedicel. The name and identification code of the female parent and the male parent, date of emasculation and pollination, time of pollination, and initials of the pollinator were recorded on the tag. The pollinated flowers were then covered with glassine bags and pinned using staples to discourage other pollinators from visiting the flower bud (Figure 2). The greenhouse was sprayed weekly using insecticides to control insects, such as ants, which are mostly attracted to nectaries, and keep flying insects from visiting the plant and accessing the flower bud.

The non-emasculated buds (self-pollinated) were covered with a glassine bag of 4 × 6 cm dimension to serve as positive controls, whereas the negative controls comprised emasculated buds without artificial pollination. Both positive and negative controls were carried out at the same time as the artificial pollination was completed. After emasculation and pollination procedures were carried out, the flower buds were keenly monitored and observed for changes that occurred from the time of pollination to pod formation, till maturity. Pod development was closely monitored for 30 days.

2.6. *Data collection and analysis*

Data were collected on the number of mature pods and number of seeds per pod. Artificial hybridization success rate was determined as per the following formula:

$$Success\ rate = (Total\ number\ of\ successful\ mature\ pods \div Total\ number\ of\ crosses\ made) \times 100$$

Data were subjected to a two-way ANOVA using the Statistical Analysis System (SAS) software version 9.4. Means were separated using Tukey's pairwise comparison at 5% level.

3. Results

3.1. *Floral architecture of C. brevidens and C. ochroleuca*

C. brevidens and *C. ochroleuca* flowers are bisexual and have papilionaceous corolla, which contains four petals that include one standard petal, two lateral wing petals and one keel petal, which cover both female and male floral organs. The stigma is globose and surrounded by anthers. Both *C. brevidens* and *C. ochroleuca* have two types of stamens, that is, long and short stamens, each comprising five anthers, for a total of 10 stamens.

3.2. *Developmental stages of C. brevidens and C. ochroleuca flower and precise flower stage for crossing*

The *C. ochroleuca* flower undergoes different developmental stages from day 1 to day 6 after bud formation. On day 1 and day 2, the flower stigma is immature and the anthers are still at the base of the bud, with closed flower petals. On day 3 and day 4, the stigma is immature with partially closed petals. The stigma is receptive on day 5 and day 6, with moderately closed flower petals. On the 6th day, the bud measures 6.0 mm and is appropriate for hand-pollination before pollen shedding. A fresh fully open flower bud is best for pollen production because pollen is mature then for pollination. The fresh fully open flower bud is observed on the 3rd day. On day 1 and day 2, the pollen is shed, though not mature enough for pollination. Self-pollination takes place between day 4 and day 5, whereas formation of the first pod begins on day 6. After successful fertilization, ovaries enlarge within 2 to 3 days and the pods are formed. If fertilization does not occur, the flower abscises or aborts after 3 days. Abortion of pods was also observed in late stages of pod development (Figure 2). Much of the pod abortion was observed between 9 and 15 days.

3.3. *Cross-pollination success*

The hybridization success between the two species was 58.33%, which was relatively higher than the negative control (0.00) but significantly lower than the positive control, which had a success rate of 81.82% because of self-pollination. The successfully hybridized plants produced a mean of 6.56 pods, which were significantly lower than those produced in the positive control, with mean number of pods = 9.00. The hybrids produced a mean of 64.86 seeds, which was significantly lower than the self-pollinated controls, which produced

Table 1. Means for pollination success rate, number of pods produced, and number of seeds for three methods of crossing *Crotalaria ochroleuca* and *C. brevidens*.

Method	Success rate (%)	Number of pods produced	Number of seeds produced
Emasculation with pollination	58.33[b†]	6.56[b]	64.86[b]
Emasculation without pollination	0.00 [c]	0.00 [c]	0.00 [c]
No artificial pollination	81.82[a]	9.00[a]	220.33[a]
Mean	46.72	5.19	95.06

†Means followed by the same letter in a column are not significantly different at $P \leq 0.05$ according to Tukey's test.

a mean of 220.33 seeds (Table 1). The combined effect of time on hybridized species showed a mean success rate of 61% for the morning crosses. A success rate of 55% was recorded for the evening crosses. The positive control had a success rate of 82%. This was also reflected in the number of pods and seeds. The mean number of pods produced by the hybrids at 8 am and 5 pm was 5.352 and 5.028, respectively, whereas the positive controls produced a mean of 9 pods (Table 2). The mean number of seeds from the hybrids in the morning was 69.25 and 60.47 in the evening, compared with 243.33 and 197.33 at 8 am and 5 pm, respectively, in the self-pollinated controls (Table 3).

4. Discussion

Pollen biotechnology is a critical component of plant reproductive biology and plays an important role in crop improvement programs. This study successfully crossed two *Crotalaria* species with distinct differences in leaf shape and pod size. Pollination success between *C. ochroleuca* and *C. brevidens* has not been previously documented; however, artificial pollination has been highly exploited in other leguminous crops, such as *Phaseolus vulgaris, Cicer arietinum, Vigna subterranea* (Free 1966; Kalve and Tadege 2017; Suwanprasert et al. 2006). Cross-pollination of *C. ochroleuca* was possible because of its flower structure.

This study explored rubbing method of crossing, as no artificial pollination method for *Crotalaria* has previously been reported. In other legumes, like common bean (*Phaseolus vulgaris* L.) and chickpea (*Cicer arietinum*) (Anbessa and Warkentin 2005), two methods (hooking and rubbing methods) are commonly used for artificial hybridization (Genchev 2007). In this particular study,

Table 2. Means ± standard error representing the effects of time of pollination on hybridization success between *Crotalaria ochroleuca* and *C. brevidens*.

Time	Success rate (%)	Number of pods produced	Number of seeds produced
8: 00 am	47.728 ± 12.83[a†]	5.352 ± 1.424[a]	104.19 ± 37.19[a]
5: 00 pm	45.708 ± 12.19[a]	5.028 ± 1.342[a]	85.94 ± 30.48[a]

†Means followed by the same letter within a column are not significantly different by Tukey's pair-wise comparison ($p < 0.05$).

Table 3. Means ± standard error (SE) for interaction between combinations and time of inter-species cross between C. ochroleuca and C. brevidens.

Method	Time	Success rate (%) (Mean ± SE)	No. of pods (Mean ± SE)	No. of seeds (Mean ± SE)
Emasculation with pollination	8am	0.61 ± 0.07	7.06 ± 0.75	69.25 ± 0.55
Emasculation with pollination	5pm	0.55 ± 0.04	6.08 ± 0.43	60.47 ± 3.39
Emasculation without pollination	8am	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Emasculation without pollination	5pm	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
No artificial pollination	8am	0.82 ± 0.10	9.00 ± 1.15	243.33 ± 29.63
No artificial pollination	5pm	0.82 ± 0.05	9.00 ± 0.58	(1) 30.31

it was not possible to use the hook method because the *Crotalaria* stigma is straight, and the bud is curved, allowing storage of lots of pollen, causing self-pollination. The stigma has a membrane that allows pollen to stick easily. The rubbing method was, therefore, found to be the most appropriate method for pollination of *Crotalaria*.

The six-day-old flower bud was found to be the best stage for emasculation and pollination. Pollination was not carried out until the 6th day, a day before pollen shedding. Choosing the correct bud for pollination determined whether or not pollination was successful. Pollination was attempted on day 4, 5, and 6. Most of the flower buds pollinated on day 4 and 5 aborted. Therefore, the developmental stage of the flower bud influenced the success rate of artificial pollination in *Crotalaria*.

Other studies indicate that apart from edaphic, biotic, and environmental factors, a major factor significantly contributing to the success of hybridization in legumes is the timing of pollination and/or crossing (Hussain et al. 2014). Various studies have shown that the final yield in different agricultural crops showed a strong relationship with the corresponding time of emasculation and pollination (Rooney 2004). Therefore, the need to standardize techniques like suitable time interval between pollination and emasculation, keeping in view the viability of pollen grain and receptivity of stigma, to increase yield and quality of final crop, is inevitable (Hussain et al. 2014).

In addition, it is important to choose the appropriate flower for pollen production. A fresh open flower (ideally 3 days after it opens) was found to have sufficient viable pollen for pollination. Pollen at this stage was yellow in color and evenly mature for pollination. This is in agreement with Veerappan et al. (2014), who discussed the developmental stages of chickpea flower. The floral biology of *C. ochroleuca* has not been previously documented. The present study reports the developmental stages of the *C. ochroleuca* flower for the first time.

The right position for handling the flower bud depended on the correct handling of forceps. Correct positioning prevented the flower from falling and minimized injury to the stigma during emasculation. Additionally, holding forceps correctly reduced the risk of injury to the pistil.

Observations from the present study indicate that for artificial pollination of *slender leaf*, the forceps should be held obliquely, pointing upward to ensure that the pollinator correctly holds the flower bud. The correct handling of flower bud in *C. ochroleuca* has not been documented; however, it has been reported in other legumes, such as beans (Free 1966; Raju and Ramana 2017).

Tagging and bagging were important precautions in the artificial pollination of *C. ochroleuca*. Tagging was done to avoid any confusion regarding the artificially pollinated flowers and the un-pollinated flowers. This technique has been previously used and documented (Massawe et al. 2003). The artificially pollinated flower in this study was covered with a glassine bag to protect it from crawling insects, such as ants, to reduce the chances of contamination by other pollination agents. Etcheverry (2001) recommended the protection of the pollinated flower bud in the greenhouse and in the field because of the presence of carpenter bees, which are common pollinators of *Crotalaria*.

Emasculation and pollination processes were carried out in the morning at 8:00 am and in the evening at 5.00 pm to determine the appropriate time for cross-pollination of *C. ochroleuca* because it has not been previously reported. However, the best time to carry out cross-pollination has been reported in other leguminous crops, such as chickpea, to be either 8.00 am or 5.00 pm (Lachyan, Desai, and Dalvi 2016). In addition, emasculation and cross-pollination in *C. juncea* have been reported, where emasculation was done at 5:00 pm and cross-pollination was done at 8:00 am the following day (Maruthi et al. 2018). In the present study, pollination of *C. ochroleuca* was done immediately after emasculation in the morning at 8.00 am and in the evening at 5.00 pm. The time of artificial pollination did not influence the success rate of pollination in *C. ochroleuca*. Therefore, this study affirms that cross-pollination of *C. ochroleuca* and *C. brevidens* can be carried out either in the morning from 8.00 am to 10.00 am or in the evening from 5.00 pm to 7.00 pm without affecting pollination-success rate.

The seed production from artificially pollinated flowers of *C. ochroleuca* was probably low because of inadequate pollen and/or low-quality pollen deposition on the stigma. Low production of seed occurs when too few pollen grains are deposited on the stigma for fertilization. Low-quality pollen deposited on stigma results in immature and low quantity of seed (Vaughton and Ramsey 2010).

The total number of pods ranged from 6 to 9 compared to seven to eleven pods per cross in the free-pollination experiment. The percentage (80%) of the seeded fruits attained from the free-pollination experiment showed that *C. ochroleuca* was self-compatible. Pod abortion mostly occurred 9 to 15 days after pod formation. Pod abortion at this stage has been observed in *C. juncea*. Thus, this observation is similar to that of Krueger et al. (2008), who reported pod abortion in a study of natural and artificial pollination of sunn hemp by insects. Poor pod maturation and high abortion rate were possibly because of pollination failure, which can occur at pre-dispersal, dispersal, or post-dispersal stage (Wilcock and Neiland 2002).

5. Conclusion

This study successfully developed an artificial interspecies-pollination protocol for *C. ochroleuca* and *C. brevidens*. Our pollination method for *Crotalaria* is easy and efficient; it involves keel petal incision of the female parent. The pollination method was successful in an inter-species cross but can also be used in intra-species crosses. The study determined that the rubbing method of pollination was ideal because of the position and structure of the stigma. A six-day-old bud was best for emasculation, whereas a fresh fully open flower was best for pollen supply. The study demonstrated that artificial pollination can be carried in the morning or evening without affecting pollination success. The method described in this study can be used as a practical guide for artificial pollination of *Crotalaria* both in the field and in the greenhouse. Successful pollination can be enhanced by obtaining the correct flower bud suitable for pollination and using the correct emasculation procedure that avoids or reduces injury to the stigma and the whole flower bud. The development of this artificial pollination protocol is an important step in hybridization and genetic improvement of the *Crotalaria* species.

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The authors declare that they have no conflict of interest.

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