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Cannabis Triploids Exhibit Reduced Fertility and Similar Growth and Flower Production Compared to Diploids

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Abstract. Cannabis (*Cannabis sativa*) grown for flowers containing cannabinoids requires all female plants, which are susceptible to seed set from exposure to pollen. Created triploids demonstrated reduced seed production compared with diploids in field and greenhouse studies in which plants were challenged with pollen from males. In the field, seed production as a percent of floral biomass ranged from 6.7% to 18.0% for triploids and from 52.6% to 57.1% for diploids. The photoperiod-insensitive triploid genotype 'Purple Star' × 'Wilhelmina' had 98.5% fewer filled (containing a developed embryo) seeds than the photoperiod-insensitive diploid genotype 'Tsunami' × 'Wilhelmina'. In the greenhouse, triploid 'Wife' had 99.5% fewer filled seeds than diploid 'Wife'. Plant growth and flower production were similar with eight triploid and seven diploid genotypes evaluated over three greenhouse studies. There were a few superior triploid and diploid genotypes; however, their performance was more likely attributable to the parental cultivar combination than ploidy level. The optimal cross direction for producing triploid seed in large quantities is tetraploid × diploid because the diploid × tetraploid cross exhibits triploid block caused by endosperm paternal excess. Colchicine-induced tetraploid parent plants should be tested over a prolonged period to eliminate cryptic chimeral mixoploids or tetraploid plants should be derived from seed produced by crossing two colchicine-induced putative tetraploid plants to ensure that seeds from tetraploid × diploid crosses will be triploid. The latter approach is necessary for photoperiod-insensitive cultivars because a prolonged period of ploidy testing is not possible for these plants. These findings indicate that triploid plants have significantly reduced fertility and are a suitable alternative to diploids in situations in which pollen exposure is possible.

Cannabis (*Cannabis sativa*) is a naturally diploid (2x=20) and dioecious species that is produced for grain, fiber, or flower for cannabinoids (Ren et al. 2021). Only female plants are grown for flower production purposes because their inflorescences contain the highest concentrations of cannabinoids. Two important and broadly marketed cannabinoids are tetrahydrocannabinol (THC) and cannabidiol (CBD) (Small 2015). In the United States, CBD-dominant cannabis is predominantly grown outdoors, whereas THC-dominant cannabis is predominantly grown indoors because of regulatory requirements (US Department of Agriculture 2022). It is anticipated that more THC-dominant cannabis production will occur outdoors because of the increasing input costs and environmental footprint from controlled indoor production (Smart et al. 2023), diminishing market value of THC-dominant flower (Demko 2022; Schoenberg 2023), greater access to dependable seed with high vigor and uniformity from breeding programs (New West Genetics 2021), and reduced availability of affordable healthy liners from clonal propagation methods (Singh 2023).

Production of cannabis flower requires the exclusion of pollen because seed set reduces cannabinoid concentrations, and colas containing seeds are unsalable as a smokable product (Feder et al. 2021; Smart et al. 2023). Unwanted pollen can originate as drift from nearby cannabis fiber and/or grain farms or from occasional male flowers on female plants (Perkowski 2019). For outdoor flower production, a crop isolation distance of 16 km is recommended to prevent detrimental cross pollination (DeDecker 2019). Part of a solution to unwanted pollen is the development of triploid (3x) cultivars that demonstrate little to no seed production ability. Triploid plants are often sterile or exhibit low fertility because they produce few to no viable gametes (Wang et al. 2016). Seedless 3x cultivars have been developed for crops such as watermelon (*Citrullus lanatus*), banana (*Musa acuminata*), hops (*Humulus lupulus*), and others (Sattler et al. 2016; Trojak-Goluch and Skomra 2013). Triploid crops may be produced by crossing tetraploid (4x) and 2x plants. The direction of the cross, 4x × 2x or 2x × 4x, can impact the quantity of viable 3x seed produced. When producing 3x watermelon, citrus (*Citrus*), banana, and hops, the optimal cross direction is 4x × 2x (Aleza et al. 2012; Silva et al. 2001; Wehner 2023), whereas the 2x × 4x cross is best for plants such as grape (*Vitis vinifera*) and tulip (*Tulipa*) (Marasek-Ciolakowska et al. 2012; Park et al. 2002).

There is only one report in the scientific literature of a single wild 4x cannabis plant, which was discovered in India (Sharma et al. 2015). Tetraploid induction of cannabis has been accomplished using mitotic inhibitors such as colchicine and oryzalin. Bagheri and Mansouri (2015) induced 4x plants by treating the apical meristem of seedlings with colchicine, and Parsons et al. (2019) did so by submerging nodal segments of in vitro shoots in a solution of oryzalin. We have produced 4x cannabis using pregerminated seed (seed with 1 to 3 mm of radicle emerged) and

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colchicine (Kurtz et al. 2020). Our method is highly effective, with 4x induction rates of 26% to 64%, depending on the cultivar, and less labor-intensive than those previously described for cannabis. The objectives of this work were to identify the optimal cross direction (4x × 2x or 2x × 4x) for producing feminized (all female) 3x seed for flower production purposes and evaluate the performance of 3x genotypes compared with 2x genotypes for seed production potential, plant growth, and flower production for cannabinoids.

Materials and Methods

Experimental cross 1. A greenhouse experiment was conducted from Nov 2020 to Feb 2021, to test the 2x × 4x cross direction. Eight 2x female plants of 'Wife' and three 2x female plants of 'Abacus' were exposed to feminized pollen from five 4x female plants of 'Kentucky Sunshine', which were masculinized using silver thiosulfate according to the protocol of Lubell and Brand (2018). Plants were grown in a greenhouse with set points of 21/17 °C day/night temperature thresholds and photoperiod control provided by supplemental lighting using 1000-W high-pressure sodium lamps (Phantom HPS 100W; Hydrofarm, Petaluma, CA, USA) and blackout curtains. A single female 4x plant of 'Kentucky Sunshine' that was not masculinized with silver thiosulfate was also grown, which served as a 4x × 4x control cross. Plants were started from cuttings from stock mother plants and potted into 7.33-L containers with peat-based medium (Metro-Mix 830; Sun Gro Horticulture, Agawam, MA, USA). Containers were top-dressed with 38 g of 15N–3.9P–10K controlled-release fertilizer (Osmocote Plus 5- to 6-month formulation; Everris NA, Dublin, OH, USA). Plants were fertigated with a 20N–8.7P–16.6K water-soluble fertilizer (Peters 20–20–20; Scotts, Marysville, OH, USA) providing 100 $mg \cdot L^{-1}$ N at each watering. Plants were grown vegetatively under an 18-h photoperiod for 35 d; then, the photoperiod was reduced to 12 h for 60 d for flowering and seed ripening. All seeds per plant were harvested and weighed. The number of seeds per plant was calculated using the weight of a random subsample of 100 seeds. Seed viability was categorized as either "filled" (brown color, round shape, and containing a well-formed embryo) or "unfilled" (tan color, round to irregular shape, and devoid of a developed embryo). The number of filled seed was counted for a random subsample of 60 seed per plant. Then, the percent filled seed per plant was calculated. The ploidy level of a subset of seeds per cross (Table 1) was determined by flow cytometry according to Kurtz et al. (2020).

Experimental cross 2. A greenhouse experiment was conducted from Jan 2021 to May 2021, to test the 4x × 2x cross direction. Five 4x female plants of 'Kentucky Sunshine' and two 4x female plants of 'Tangerine' were exposed to feminized pollen from two 2x female plants of 'Wife', which were masculinized as described. Two female 2x plants each of 'Wife' and 'Abacus' were also grown to serve as 2x × 2x control crosses. All plants were started from cuttings from stock mother plants, except for the two 'Tangerine' plants, which were each from the original seeds treated with colchicine. Greenhouse conditions were as described for experimental cross 1, except that plants were grown under an 18-h photoperiod for 30 d, and then under a 12-h photoperiod for 71 d. Containers, potting medium, fertilizer applications, and data collection were as described.

Other crosses. Eight other crosses, six that were 4x × 2x and two that were 4x × 4x, were conducted for the primary purpose of producing additional 3x feminized seed for the seed production and plant growth and flower production studies. Crosses were conducted in a greenhouse as described or growth chamber with a set point of 24 °C and photoperiod control provided by light-emitting diode lamps at 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$. All plants were started from seed, except for 2x and 4x 'Wife', which were started from cuttings from stock mother plants. All parent plants, except for 2x and 4x 'Wife', were photoperiod-insensitive cultivars. Plants were potted in 2.68-L or 7.33-L containers, depending on the cultivar, filled with the same potting medium as described, and top-dressed with the same controlled-release fertilizer as described at 10 g or 38 g, respectively. Pollinations were conducted by hand to the greatest extent possible using fresh pollen from masculinized plants grown simultaneously alongside maternal plants or stored pollen from plants masculinized at an earlier date. Pollen to be stored was spread out on paper and left at room temperature for 24 h to dry, collected into plastic tubes, and set at −20 °C for storage. Data collection was performed as described previously.

Seed production studies. A greenhouse study was conducted from Sep 2021 to Dec 2021, to compare the seed production ability of one 2x female genotype of 'Wife' and two 3x female genotypes of 'Wife' (Table 2). Plants were started from cuttings from stock mother plants, potted into containers, and fertilized as described for experimental cross 1. The experimental unit (EU) was a single potted plant, and plants were arranged in a randomized complete block design (RCBD) with 11 replications. Plants were grown under an 18-h photoperiod for 14 d, and then under a 12-h photoperiod for 62 d. Pollen was provided by 11 2x male plants of 'Youngsim10' that were started from seed and potted as described. Male plants were grown in the same greenhouse as female plants, but they were not randomized with female plants. Hand pollination was conducted for 12 terminal inflorescences per female plant. Additionally, pollen was dispersed by air flow from greenhouse fans and venting. Male plants were discarded after anthesis. On day 56 of the study, plant height, number of shoots, and total shoot length were measured per plant. All seeds per plant were harvested. For 3x plants, all seed produced was counted; for 2x plants, the number of seed produced per plant was estimated using the weight of a random subsample of 100 seeds. The number of filled seeds was determined for a random subsample of 60 seed, and this value was used to calculate the percent filled seed per plant. Seed caliper was measured for eight filled seed per plant. The cannabinoid content of dry flower was analyzed by high-performance liquid chromatography at the University of Connecticut Center for Environmental Sciences and Engineering in Storrs, CT, USA.

A field study was conducted from May 2022 to Oct 2022, to compare the seed production ability of four 2x and five 3x female genotypes (Table 3). Male 2x plants of 'Purple Star' × 'Youngsim10' and 'Youngsim10' were included in the field planting to provide pollen. Female plants were started from seed and male plants from cuttings from stock plants. Plants were potted in 1.04-L containers in the same potting medium, as described, and grown in a greenhouse under an 18-h photoperiod for 24 d before transplanting to the field on 31 May 2022. The ploidy level of plants was confirmed by flow cytometry before transplanting in the field. The field planting was located at the University of Connecticut Plant Science Research and Education Facility in Storrs,

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Table 1. Plant description and ploidy level (4x= tetraploid; 3x= triploid, 2x=diploid, 4x+2x = mixoploid) of cultivars of *Cannabis sativa* used as parent plants in crosses and progeny from crosses.

Maternal plant	Maternal plant ploidy at time of crossing	Paternal plant	Paternal plant ploidy at time of crossing	Cross location	Maternal plant (no.)	Avg seeds per maternal plant (no.)	Avg percent filled seeds per maternal plant[i]	Total progeny tested by flow cytometry (no.)	Progeny at each ploidy level, no. (% of tested)			Actual 4x parent(s) ploidy	4x parent(s) ploidy determination[ii]
									2x	3x	4x		
Experimental cross 1													
Wife	2x	Kentucky Sunshine	4x	Greenhouse	8	3775	0.3	22	10 (45)	12 (55)	0	4x	Stable 4x from colchicine
Abacus	2x	Kentucky Sunshine	4x	Greenhouse	3	791	0.4	11	3 (27)	8 (73)	0	4x	Stable 4x from colchicine
Kentucky Sunshine	4x	Kentucky Sunshine	4x	Greenhouse	1	1113	76.1	23	0	0	23	4x	Stable 4x from colchicine
Experimental cross 2													
Kentucky Sunshine	4x	Wife	2x	Greenhouse	5	63	76.2	60	0	54 (90)	6 (10)	4x	Stable 4x from colchicine
Wife	2x	Wife	2x	Greenhouse	2	192	72.9	10	10 (100)	0	0	n/a	n/a
Abacus	2x	Wife	2x	Greenhouse	2	520	93.5	10	10 (100)	0	0	n/a	n/a
Tangerine	4x	Wife	2x	Greenhouse	2	44	56.8	20	9 (45)	11 (55)	0	4x+2x	Cryptic chimeral mixoploid from colchicine
Other crosses													
Wife	4x	Purple Star	2x	Growth chamber	2	1557	65.5	52	0	51 (98)	1 (2)	4x	Stable 4x from colchicine
Purple Star	4x	Wilhelmina	2x	Growth chamber	3	97	84.5	32	1 (3)	31 (97)	0	4x	Stable 4x from seed
Purple Star	4x	Purple Star	2x	Greenhouse	4	13	n/a	25	0	25 (100)	0	4x	Stable 4x from seed
Purple Star	4x	Wife	2x	Greenhouse	4	127	84.0	44	0	30 (68)	14 (32)	4x	Stable 4x from seed
Wilhelmina	4x	Wife	2x	Growth chamber	1	175	75.4	20	15 (75)	4 (20)	1 (5)	4x+2x	Cryptic chimeral mixoploid from colchicine
Tsunani	4x	Wife	2x	Growth chamber	2	546	53.9	63	8 (13)	41 (65)	14 (22)	4x+2x	Cryptic chimeral mixoploid from colchicine
Purple Star	4x	Purple Star	4x	Growth chamber	5	519	88.6	25	0	0	25 (100)	4x	Stable 4x from seed
Wilhelmina	4x	Wilhelmina	4x	Growth chamber	1	479	82.0	24	4 (17)	7 (29)	13 (54)	4x+2x	Cryptic chimeral mixoploid from colchicine

[i] Filled seed = brown in color, round in shape, and containing a well-formed embryo; unfilled seed = tan in color, round to irregular in shape, and devoid of a developed embryo.
[ii] Stable 4x from colchicine = tested 4x 10 times over 140 d; cryptic chimeral mixoploid from colchicine = tested 4x initially, but later tested 4x+2x; stable 4x from seed = derived from 4x × 4x cross and tested 4x.
n/a = not applicable.

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Table 2. Number of seeds, filled seeds (brown color, round shape, and containing a well-formed embryo), unfilled seeds (tan color, round to irregular shape, and devoid of a developed embryo), seed caliper, plant height, number of shoots per plant, total shoot length per plant, percent cannabidiol (CBD), and percent tetrahydrocannabinol (THC) from diploid (2x) and triploid (3x) *Cannabis sativa* 'Wife' grown in a greenhouse.

Genotype	Ploidy	Seeds (no.)	Filled seeds (%)	Unfilled seeds (%)	Seed caliper (mm)	Plant ht (cm)	Shoots per plant (no.)	Total shoot length (cm) per plant	CBD (%)	THC (%)
Wife (2x)	2x	3010.3 a[i]	88.5 a	11.5 c	4.6 b	98.5 a	19.0 a	829.3 a	7.4 a	0.37 a
Wife-1 (4x) × Wife (2x)	3x	99.0 b	8.8 b	91.2 a		66.2 b	10.0 b	375.5 b	6.0 b	0.26 b
Wife-6 (4x) × Wife (2x)	3x	118.3 b	14.2 b	85.8 b	5.2 a	71.2 b	13.0 b	441.0 b	7.4 a	0.38 a

[i] Mean separation within columns (indicated by different letters) according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$ (percent CBD and percent THC, n = 5; all others, n = 11).

CT, USA (lat. 41.79544°N, long. −72.22836°W). The field soil was a Paxton and Montauk fine sandy loam with 6.7% organic matter and a pH 5.9. The EU was a single plant, and plants were arranged as an RCBD with 10 replications. Each block included 10 genotypes, nine female genotypes (five 3x and four 2x), and one male genotype, either 'Purple Star' × 'Youngsim10' or 'Youngsim10', such that five blocks had 'Purple Star' × 'Youngsim10' and five blocks had 'Youngsim10'. Plants were grown in rows with 1.8-m spacing on-center between rows and 1.2-m spacing on-center within rows. To facilitate manual irrigation, two blocks were established per planting row; therefore, there was a total of five rows. To ensure that pollen presence was strong, an additional male plant of 'Youngsim10' was installed per planting row between the two blocks, thus forming a row of five male plants that bisected the planting. A total of 105 plants were installed; of these, 90 were female and 15 were male (10 plants of 'Youngsim 10' and five plants of 'Purple Star' × 'Youngsim10'). Plants were fertilized twice, at the time of planting and again on 7 Jul 2022, with 10 g of granular fertilizer (All Purpose 10N–4.4P–8.3K; Greenview, Lebanon, PA, USA) per plant each time. Fertilizer was broadcast around the base of the plant by hand. Plants were irrigated by hand as needed throughout the growing season. Weeding was performed by rototiller between rows and by hand within rows. Peak anthesis was noted for male plants when ∼50% of flowers were open based on visual observation of inflorescences. Plants were harvested when seeds appeared ripe and ∼50 d after the onset of terminal flowering (when a minimum of three pistils bearing stigmas were visible at the shoot tips) according to Spitzer-Rimon et al. (2019), which varied by genotype. For photoperiod-sensitive genotypes, four terminal inflorescences per plant (as the subsample) were harvested, dried in an air-circulating oven at 45 °C for 24 h, weighed, and underwent seed removal. Terminal inflorescences were 20 to 25 cm long when measured from the terminal apex of the shoot inward. Seeds were weighed and the seed

Table 3. Seed production as a percent of floral biomass, number of seeds per plant, and percent filled seeds (brown color, round shape, and containing a well-formed embryo) of diploid (2x) and triploid (3x), photoperiod-sensitive genotypes and photoperiod-insensitive genotypes of *Cannabis sativa* grown in a field.

Genotype	Ploidy	Photoperiod sensitivity	Sampled dry floral biomass (g)	Seed wt (g) of sample	Seed production (% of floral biomass)	Seeds (no.)	Filled seeds (%)	Total progeny tested by flow cytometry (no.)	Progeny, no. (% of progeny tested)	
									2x	3x
Abacus (2x) × Wife (2x)	2x	Sensitive	66.8	38.1	57.1 a[i]		86.1 a			
Purple Star (2x) × Wife (2x)	2x	Sensitive	102.4	53.6	52.6 a		87.5 a			
Tsunami (2x) × Wilhelmenia (2x)	2x	Insensitive		19.8		1803.2 a	75.5 a			
Wife (2x) × Wife (2x)	2x	Sensitive	48.9	26.5	54.5 a		86.7 a			
Kentucky Sunshine (4x) × Wife (2x)	3x	Sensitive	45.1	4.3	10.1 c		26.5 bc	30	13 (43)	17 (57)
Purple Star (4x) × Wife (2x)	3x	Sensitive	74.3	5.2	6.7 c		34.0 b	30	14 (47)	16 (53)
Purple Star (4x) × Wilhelmenia (2x)	3x	Insensitive		0.2		26.6 b	16.6 c			
Tsunami (4x) × Wife (2x)	3x	Sensitive	109.6	19.6	18.0 b		30.9 bc	30	14 (47)	16 (53)
Wife (4x) × Purple Star (2x)	3x	Sensitive	85.0	11.1	13.0 bc		29.9 bc	30	19 (63)	11 (37)

[i] Mean separation within columns (indicated by different letters) according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$ (n = 10).

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Table 4. Plant height, number of shoots, total shoot length, stem caliper, plant dry weight, floral dry weight, percent flower dry weight, percent cannabidiol (CBD), and percent tetrahydrocannabinol (THC) of diploid (2x) and triploid (3x) genotypes of cannabis (*Cannabis sativa*) grown in a greenhouse.

Genotype	Ploidy	Plant ht (cm)	Shoots (no.)	Total shoot length (cm)	Stem caliper (mm)	Plant dry wt (g)	Flower dry wt (g)	Flower dry wt (%)	CBD (%)	THC (%)
Abacus	2x	62.8 c[i]	10.5 c	468.8 ed	10.9 c	102.7 ab	53.3 ab	52.5 a	11.9 ab	0.47 a
Kentucky Sunshine	2x	80.3 bc	14.5 abc	576.5 cde	12.9 bc	115.9 ab	42.4 ab	36.8 bc	9.2 cde	0.29 b
Tangerine	2x	99.2 ab	16.5 a	724.0 abc	11.7 c	148.9 ab	67.6 a	44.8 ab	7.9 de	0.24 b
Wife	2x	111.3 a	16.0 a	732.3 abc	12.9 c	99.3 ab	32.5 ab	33.3 c	10.2 bcd	0.40 a
Abacus (2x) × Kentucky Sunshine (4x)	3x	121.5 a	15.3 ab	843.3 a	16.8 ab	176.9 a	60.7 ab	35.3 bc	14.2 a	0.48 a
Tangerine (2x) × Kentucky Sunshine (4x)	3x	83.5 bc	14.5 abc	628.8 bcd	14.7 abc	116.4 ab	38.3 ab	32.5 c	8.5 ed	0.27 b
Wife (2x) × Kentucky Sunshine (4x)	3x	115.0 a	14.8 ab	774.8 ab	17.2 a	166.1 ab	47.9 ab	27.8 c	11.4 bc	0.40 a
Wife (4x) × Wife (2x)	3x	75.8 bc	11.5 bc	441.0 e	12.6 c	71.6 b	24.4 b	34.0 bc	7.3 e	0.29 b

[i] Mean separation within columns (indicated by different letters) according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$ (percent CBD and THC, n = 3; all others, n = 4).

percent of floral biomass was calculated. The number of filled seeds per plant was determined using a random subsample as described, and the percent filled seed was calculated. For the two photoperiod-insensitive genotypes, the entire plant was harvested by cutting at the stem base, and all seeds per plant were counted. The ploidy level of 30 filled seeds selected at random per 3x photoperiod-sensitive genotype was determined using flow cytometry.

Growth and flower production studies. Three separate greenhouse studies were conducted to compare 2x and 3x genotypes for growth and flower production. Greenhouse set points were as described, and the ploidy level of plants was confirmed by flow cytometry at the start of each study. The first study was conducted from May 2021 to Jul 2021, and included four 3x and four 2x genotypes (Table 4). Plants were started from cuttings from stock mother plants and potted into 7.33-L containers as described. The EU was a single potted plant, and plants were arranged as an RCBD with four replications. Plants were grown under an 18-h photoperiod for 10 d, and then under a 12-h photoperiod for 42 d. On day 38 of the study, plant height, number of shoots, and total shoot length per plant were measured. On day 41 of the study, the stem caliper measured at 3 cm above the medium surface was recorded per plant. At harvest, plants were cut at the stem base, allowed to dry at room temperature for 14 d, and weighed; then, flowers were separated from stems and leaves and weighed. The percent flower weight was calculated by dividing the flower dry weight by the plant dry weight and multiplying by 100%. The cannabinoid content of dry flower was analyzed as described.

The second greenhouse study was conducted from Aug 2021 to Dec 2021, and included three 2x and four 3x genotypes (Table 5). Plants were started from seed and potted into containers as described for greenhouse study 1. The EU was a single potted plant, and plants were arranged as an RCBD with six replications. Plants were grown under an 18-h photoperiod for 35 d, then under a 15-h photoperiod for 21 d, and then under a 12-h photoperiod for 30 d. The critical photoperiod varied for the genotypes used in this study; therefore, plants were harvested at 3 weeks after the onset of terminal flowering, noted as described and according to Spitzer-Rimon et al. (2019). Data collection was conducted as described for greenhouse study 1. On day 57 of the study, the stem caliper was measured as described. On day 67 of the study, plant height, the number of shoots, and the total shoot length were measured.

The third greenhouse study was conducted from Oct 2022 to Nov 2022, and included 2x and 3x genotypes for three parental cross combinations (Table 6). Plants were started from seed and potted into containers as described for greenhouse study 1. The EU was a single potted plant, and plants were arranged as an RCBD with eight replications. Plants were grown under an 18-h photoperiod for 28 d, and then under a 12-h photoperiod for 44 d. Plant harvest and data collection were conducted as described for greenhouse study 1. The plant height, number of shoots, total shoot length, and stem caliper were measured on day 60 of the study. In addition to the cannabinoid content, the terpene content of dry flower was analyzed by gas chromatography at the University of Connecticut Center for Environmental Sciences and Engineering.

Statistical analysis. Data were subjected to an analysis of variance (PROC GLIMMIX) and mean separation with Tukey's honestly significant difference test ($P \leq 0.05$) using statistical software (SAS version 9.4; SAS Institute, Cary, NC, USA). Interaction effects (counterpart × ploidy level) were reported for growth and flower production in greenhouse study 3 because they were significant for some dependent variables.

Results and Discussion

High levels of seed formation were observed on 2x plants pollinated with 4x feminized pollen in experimental cross 1; however, >99.5% of seed failed to develop and were devoid of an embryo (unfilled) (Table 1, Fig. 1A). The 4x × 4x cross conducted simultaneously using the same pollen source was successful, which suggested that the problem was the 2x × 4x cross direction, not unviable pollen. Crawford et al. (2021) also reported empty or abnormal feminized seed production from the 2x × 4x cross for cannabis.

It is worth noting the observed high levels of feminized seed production from 4x cannabis because it has been reported that 4x cannabis has dramatically reduced fertility (Crawford et al. 2021; Smart et al. 2023). Others reported that created 4x plants

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Table 5. Plant height, number of shoots, total shoot length, stem caliper, plant dry weight, flower dry weight, percent flower dry weight, percent cannabidiol (CBD), and percent tetrahydrocannabinol (THC) of diploid (2x) and triploid (3x) photoperiod-sensitive and photoperiod-insensitive genotypes of cannabis (*Cannabis sativa*) grown in a greenhouse.

Genotype	Ploidy	Photoperiod sensitivity	Plant ht (cm)	Shoots (no.)	Total shoot length (cm)	Stem caliper (mm)	Plant dry wt (g)	Flower dry wt (g)	Flower dry wt (%)	CBD (%)	THC (%)
Purple Star (2x) × Purple Star (2x)	2x	Insensitive	45.0 c[i]	7.0 d	180.3 c	10.3 c	35.0 c	9.1 d	25.9 a		
Purple Star (2x) × Wife (2x)	2x	Sensitive	114.5 ab	22.2 a	1199.3 a	21.8 a	478.3 a	42.8 bc	8.9 c	9.9 a	0.37 a
Wife (2x) × Wife (2x)	2x	Sensitive	105.3 b	20.0 bc	936.3 b	17.0 b	401.7 ab	50.6 b	12.6 bc	6.3 b	0.22 b
Kentucky Sunshine (4x) × Wife (2x)	3x	Sensitive	127.2 ab	21.2 ab	1121.7 a	21.2 a	451.7 a	69.4 a	15.4 bc	9.5 a	0.33 a
Purple Star (4x) × Purple Star (2x)	3x	Insensitive	58.0 c	7.0 d	222.0 c	10.1 c	38.1 c	7.9 d	20.7 ba		
Purple Star (4x) × Wife (2x)	3x	Sensitive	139.4 a	19.0 bc	1107.2 ab	19.0 ab	336.7 b	45.9 bc	13.6 bc	8.3 ab	0.30 ab
Wife (4x) × Purple Star (2x)	3x	Sensitive	116.3 ab	18.7 c	951.0 b	18.8 ba	378.3 ab	32.0 c	8.5 c	6.4 b	0.30 ab

[i] Mean separation within columns (indicated by different letters) according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$ (plant dry weight, flower dry weight, percent flower dry weight, percent CBD, and percent THC, n = 3; all others, n = 6).

were completely fertile, with larger seeds and seed yields (Bocsa 1999; Warmke and Davidson 1944). The 'Kentucky Sunshine' (4x) mother plant in experimental cross 1 was 1 m tall and wide and produced 1113 seeds; of these, 76.1% contained a developed embryo (filled seed) (Table 1, Fig. 1B). In another 4x × 4x cross, 'Purple Star' (4x) plants produced more than 500 seeds per plant (88.6% filled), which was a surprisingly large amount because this plant exhibits a dwarf habit and is photoperiod-insensitive. In a 4x × 2x cross conducted between 'Wife' (4x) and 'Purple Star' (2x), more than 1500 seeds (65.5% filled) were produced per plant (Fig. 2). Crawford et al. (2021) compared the 2x × 2x and 4x × 4x for the same parental cultivar cross (all female parents) and found that the 2x parent produced 317.25 filled seeds per plant and the 4x parent produced 124.75 filled seeds per plant. These observations suggest that the fertility level of 4x cannabis can vary based on genotype, as has been found for other plant species (Brand and Durocher 2022; Kuckuck and Levan 1951), and that it may be possible to identify highly fertile 4x plants through mass selection.

When 'Kentucky Sunshine' (4x) was pollinated with 'Wife' (2x) in experimental cross 2, the resulting seed was 76.2% filled. Based on a tested subsample, 90% of seeds produced were 3x;

Table 6. Plant height, number of shoots, total shoot length, stem caliper, plant dry weight, flower dry weight, percent flower dry weight, percent cannabidiol (CBD), and percent tetrahydrocannabinol (THC) of photoperiod-sensitive and photoperiod-insensitive diploid (2x) and triploid (3x) counterparts from the same parental cannabis (*Cannabis sativa*) cultivar crosses Purple Star × Purple Star, Purple Star × Wife, and Tsunami × Wife grown in a greenhouse.

Genotype	Ploidy	Photoperiod sensitivity	Plant ht (cm)	Shoots (no.)	Total shoot length (cm)	Stem caliper (mm)	Plant dry wt (g)	Flower dry wt (g)	Flower dry wt (%)	CBD (%)	THC (%)
Purple Star (2x) × Purple Star (2x)	2x	Insensitive	47.0 a[i]	5.9 a	160.6 a	7.9 a	23.6 a	7.1 a	30.0 b	8.8 a	0.36 a
Purple Star (4x) × Purple Star (2x)	3x	Insensitive	51.8 a	6.3 a	186.0 a	8.0 a	24.2 a	8.0 a	33.7 a	8.9 a	0.38 a
Purple Star (2x) × Wife (2x)	2x	Sensitive	85.3 a	15.6 a	706.4 a	14.1 a	138.6 a	29.4 a	19.9 a	11.8 a	0.53 a
Purple Star (4x) × Wife (2x)	3x	Sensitive	98.7 a	14.0 a	660.0 a	13.7 a	155.0 a	26.5 a	16.7 a	9.9 a	0.44 a
Tsunami (2x) × Wife (2x)	2x	Sensitive	117.4 a	17.6 a	1026.8 a	20.0 a	272.5 a	54.4 a	24.1 a	9.5 a	0.40 a
Tsunami (4x) × Wife (2x)	3x	Sensitive	110.2 a	14.6 b	788.5 b	18.4 a	212.9 a	36.5 a	17.1 b	9.7 a	0.40 a

[i] Mean separation within columns within counterparts (indicated by different letters) according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$ (CBD and percent THC, n = 4; all others, n = 8).

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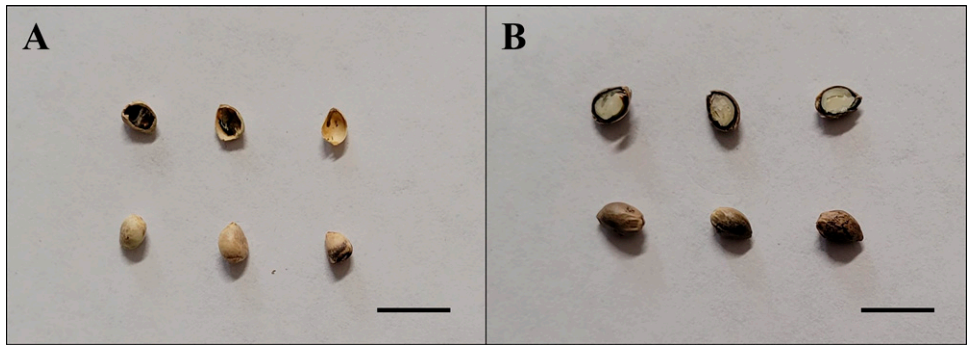

Fig. 1. Representative seeds of cannabis (*Cannabis sativa*). Cut seeds are displayed in the top row and intact seeds are displayed in bottom row. (**A**) Unfilled seeds = tan color, round to irregular shape, and devoid of a developed embryo. (**B**) Filled seeds = brown color, round shape, and containing a well-formed embryo. Scale bars = 1 cm.

however, a small number of seeds (10%) were 4x. A small number of 4x progeny were also observed for 4x × 2x crosses between 'Purple Star' (4x) × 'Wife' (2x), 'Tsunami (4x) × 'Wife' (2x), 'Wife' (4x) × 'Purple Star (2x), and 'Wilhelmina' (4x) × 'Wife' (2x). The 4x seed likely resulted from solitary male flowers on the maternal plants that were not discovered before anthesis or 2x gamete formation in the 2x parent (Fig. 3). Similarly, occasional male flowers on maternal 'Wife' (2x) plants in experimental cross 1 resulted in 2x seed. There was less seed production than expected in experimental cross 2 because plants experienced accidental drying of potting medium during floral development, which killed three of the five 'Wife' (2x) plants being masculinized with silver thiosulfate to provide pollen.

Our findings indicated that the 4x × 2x is the optimal cross direction for producing 3x feminized seed of cannabis. The 2x × 4x cross is not a viable option for producing 3x seed because of triploid block resulting from arrested endosperm development after fertilization (Stoute et al. 2012). Endosperm development may be impacted by the ratio of maternal to paternal (M:P) ploidy contributions to the endosperm. For the natural 2x × 2x cross and the 4x × 4x cross, the endosperm ploidy ratio is 2M:1P; however, they are 4M:1P and 2M:2P for the 4x × 2x cross and 2x × 4x cross, respectively. When the maternal contribution exceeds the paternal contribution in 2x × 2x, 4x × 4x, and 4x × 2x crosses, endosperm and embryo development proceeds and filled seeds are produced. However, for 2x × 4x crosses, the maternal and paternal contributions are equivalent at 2M:2P (a scenario referred to as paternal excess), and endosperm development does not occur (Batista et al. 2019). Without the presence of endosperm to nourish the growing embryo, the embryo fails to expand and fill the seed (Ram 1960). Arrested endosperm development caused by paternal excess has been reported for cabbage (*Brassica oleracea*), mouse-ear cress (*Arabidopsis thaliana*), and potato (*Solanum* sp.) (Batista et al. 2019; Carputo et al. 1999; Stoute et al. 2012).

When inducing tetraploids with colchicine, mixoploid individuals (with both 2x and 4x cells) will occur with some regularity depending on the induction method and cultivar (Bagheri and Mansouri 2015; Kurtz et al. 2020). Mixoploid (2x+4x) plants are usually identified by flow cytometry and eliminated. However, 2x+4x individuals may be mistakenly identified as 4x when the number of 2x cells is too low to detect and the plant is tested while young. Surviving 2x cells can multiply to occupy a section of the apical meristem forming a sectorial chimera (Juneja and Gopal 1960). We refer to individuals that originally test as 4x but later test 2x+4x as cryptic chimeral mixoploids. A prolonged period of repeated testing by

Fig. 2. Tetraploid maternal plants of cannabis (*Cannabis sativa*) cultivar Wife in 7.33-L containers and inside a growth chamber that were crossed with diploid 'Purple Star' to produce feminized triploid seeds.

Fig. 3. A solitary male flower on a female cannabis (*Cannabis sativa*) plant.

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flow cytometry is required to ensure that a 4x is stable from colchicine, not a cryptic chimeral mixoploid. The ploidy levels of 'Wife' (4x) and 'Kentucky Sunshine' (4x) were tested 10 times over 140 d to establish that they were stable 4x from colchicine. 'Tangerine' plants in experimental cross 2 were not tested over a sufficiently long period, and they eventually revealed themselves to be 2x+4x (Table 1). The resulting seed crop from 'Tangerine' was a mix of 2x and 3x progeny, which would be unacceptable for a seed producer who wants to provide quality 3x seed to growers.

Photoperiod-insensitive cultivars are generally small in stature and yield less than photoperiod-sensitive cultivars do; however, they are desirable to growers and plant breeders because of their fast production cycle (Coolong et al. 2023). Furthermore, research has shown that photoperiod-insensitive plants may be used to breed early-flowering 2x and 3x cultivars (Kurtz et al. 2023), which would benefit flower producers (Stack et al. 2021). There is a short period when the ploidy of putative photoperiod-insensitive 4x can be evaluated because they initiate flowering within 21 to 35 d of germination and cannot be maintained vegetatively. The short evaluation time for putative tetraploids proved insufficient to eliminate cryptic chimeral mixoploids of photoperiod-insensitive 'Wilhelmina' and 'Tsunami' parents (Table 1).

With any colchicine-induced 4x plant, there is the risk of residual 2x cells that were not converted to 4x. These 2x cells can remain unidentified and will disrupt the 3x seed breeding process. To avoid this risk, we recommend the following breeding strategy to develop parent plants that are 100% 4x: cross two putative colchicine-induced 4x plants, raise seed, use flow cytometry to identify nonchimeral 4x progeny (from the fusion of 2x egg and 2x sperm), and use these 4x progeny as breeding parents. For example, when two cryptic chimeral mixoploid 'Wilhelmina' plants were crossed, 2x, 3x, and 4x progeny were produced (Table 1). Based on a tested subsample, 54% of the progeny were fully 4x, and these plants could be reliably used as 4x parents for breeding. Fully 4x genotypes may be crossed with 2x plants to produce 100% 3x seed populations and/or crossed with 4x plants to produce 100% 4x seed populations as stock for future breeding needs. For example, when seed-derived 'Purple Star' (4x) served as maternal plants in crosses with 'Purple Star' (2x) and 'Wilhelmina' (2x), the progeny from both crosses were 3x (Table 1). From the latter cross, one 2x individual was identified, which may represent an aneuploid or anomaly of flow cytometry. When seed-derived 'Purple Star' (4x) plants were crossed with each other, the progeny was 4x (Table 1). Although it was not a study objective, we observed better seed production when crosses were conducted using a growth chamber instead of the greenhouse, probably because a growth chamber provides consistent light and temperature, both of which impact irrigation demand.

In the greenhouse, 3x genotypes of 'Wife' produced ~99.5% fewer filled seeds than 2x 'Wife' did (Table 2). Seed produced by plants of 3x genotype 'Wife'-6 (4x) × 'Wife' (2x) had a larger caliper than those produced by 'Wife' (2x). There were not enough filled seeds produced by plants of 3x genotype 'Wife'-1 (4x) × 'Wife' (2x) to measure the caliper. The percent CBD and percent THC of 3x genotypes of 'Wife' were the same or slightly lower than those of 2x 'Wife'. Crawford et al. (2021) tested the seed production ability of 3x and 2x plants of the same cultivar cross, 'TS1–3' × 'P163', using feminized pollen. They report 4.25 filled seeds per 3x plant and 317.25 filled seeds per 2x plant. The levels of seed production were lower than those of our greenhouse study, likely because feminized pollen is less viable than pollen from genetically male plants (DiMatteo et al. 2020).

In the field, pollen was provided by the male 2x genotypes 'Purple Star' × 'Youngsim10' and 'Youngsim10'. Anthesis for 'Purple Star' × 'Youngsim10' ranged from 22 Jun to 31 Aug, with peak anthesis from 6 Jul to 3 Aug. Anthesis for 'Youngsim10' ranged from 17 Aug to 20 Sep, with peak anthesis from 31 Aug to 14 Sep. Plants of genotype 'Purple Star' × 'Youngsim10' flowered earlier than plants of 'Youngsim10' because this genotype had a photoperiod-insensitive parent, 'Purple Star' (Kurtz et al. 2023). The period of anthesis for the two male genotypes overlapped for 14 d, from 17 to 31 Aug. The male plants in the planting provided a continuous supply of pollen over an 84-d period, which covered anthesis for all nine female genotypes in the study. Seed production as a percent of floral biomass ranged from 52.6% to 57.1% for 2x photoperiod-sensitive genotypes, and 86.1% to 87.5% of seeds were filled (Table 3). Seed production from 3x photoperiod-sensitive genotypes was significantly less, at 6.7% to 18.0% of floral biomass, and 26.5% to 34.0% of seeds were filled. For the photoperiod-insensitive genotypes, 3x plants of genotype 'Purple Star' (4x) × 'Tsunami' (2x) produced 98.5% fewer filled seeds than 2x plants of genotype 'Tsunami' (2x) × 'Wilhelmina' (2x). According to seed producers, yields of 1000 seeds per plant from photoperiod-insensitive 2x cultivars are supra-optimal (Davidoff A, Founder, Atlas Seed, Sebastopol, CA, USA, personal communication). Plants of photoperiod-insensitive 2x genotype 'Tsunami (2x) × 'Wilhelmina' (2x) produced approximately 1300 filled seeds per plant, indicating that the study plants were well-challenged with pollen. In reality, a flower crop is unlikely to experience the heavy pollen exposure provided during the study planting. Flower growers reported that unexpected male flowers in a crop can result in 50 to 200 seeds per plant, thus rendering the inflorescences unsalable as smokeable product. An acceptable rate is 10% of this value or 5 to 20 seeds per plant (Davidoff A, Atlas Seed, personal communication). Photoperiod-insensitive 3x genotype 'Purple Star' (4x) × 'Tsunami' (2x) produced approximately five filled seeds per plant. It is probable that other 3x cultivars developed will demonstrate similar seed production when exposed to unexpected pollen. In the field planting, approximately equivalent amounts of 2x and 3x progeny were produced per 3x genotype (Table 3). This finding was not surprising because meiosis in triploids results in unbalanced segregation of chromosomes and aneuploid gametes (St Charles et al. 2010). Owen (2023) evaluated the ploidy of 92 seedlings from the 3x cultivars Suver Haze Seedless, Sour Suver Haze Seedless, and White CBG Seedless and found that most of them (>92%) were 2x.

Seven 2x genotypes and eight 3x genotypes were evaluated to determine multiple plant growth and flower traits over three greenhouse studies (Tables 4–7). Overall, 2x and 3x genotypes performed similarly, with a few exceptions. During the first study, 3x plants of 'Abacus' (2x) × 'Kentucky Sunshine' (4x) and 'Wife' (2x) × 'Kentucky Sunshine' (4x) had larger stem calipers than most 2x plants (Table 4). The CBD content of 3x genotype 'Abacus' (2x) × 'Kentucky Sunshine (4x) was greater than that of other genotypes, except its 2x parent 'Abacus' (Table 4). 'Abacus' (2x) also had a greater percent flower weight than other genotypes, except 'Tangerine' (2x). Fernandes et al. (2023) also observed a cultivar-dependent response to increasing ploidy of cannabis for the plant height and leaf size traits. During the second study, 2x and 3x plants of 'Purple Star' were the smallest, which was not unexpected because they are photoperiod-insensitive

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Table 7. Terpene content (ug·g^{-1}) of photoperiod-sensitive and photoperiod-insensitive diploid (2x) and triploid (3x) counterparts from the same parental cannabis (*Cannabis sativa*) cultivar crosses Purple Star × Purple Star, Purple Star × Wife, and Tsunami × Wife grown in a greenhouse.

Genotype	Ploidy	Photoperiod sensitivity	α-Pinene (ug·g^{-1})	β-Pinene (ug·g^{-1})	β-Myrcene (ug·g^{-1})	d-Limonene (ug·g^{-1})	Terpinolene (ug·g^{-1})	Linalool (ug·g^{-1})	β-Caryophyllene (ug·g^{-1})	α-Humulene (ug·g^{-1})
Purple Star (2x) × Purple Star (2x)	2x	Insensitive	47.7 a[1]	86.7 a	717.7 a	194.1 a	811.5 a	82.4 a	1126.4 a	437.3 a
Purple Star (4x) × Purple Star (2x)	3x	Insensitive	57.2 a	103.2 a	504.9 a	219.7 a	1038.6 a	94.5 a	1144.9 a	470.6 a
Purple Star (2x) × Wife (2x)	2x	Sensitive	101.7 a	170.4 a	193.0 b	226.8 a	817.5 a	172.8 a	1081.5 b	411.6 a
Purple Star (4x) × Wife (2x)	3x	Sensitive	38.6 b	76.2 b	657.9 a	196.6 a	262.1 b	237.5 a	2353.6 a	354.1 a
Tsunami (2x) × Wife (2x)	2x	Sensitive	47.3 a	55.0 a	266.9 a	221.0 a	15.5 a	166.1 b	921.0 a	503.5 a
Tsunami (4x) × Wife (2x)	3x	Sensitive	29.7 a	50.4 a	153.7 a	244.9 a	24.6 a	261.4 a	1085.7 a	553.3 a

[1] Mean separation within columns within counterparts (indicated by different letters) according to Tukey's honestly significant difference (HSD) test at $P \leq 0.05$ (n = 4).

Fig. 4. Diploid and triploid counterparts of cannabis (*Cannabis sativa*) from the parental cultivar crosses Purple Star × Purple Star, Purple Star × Wife, and Tsunami × Wife. Scale bars = 20 cm.

(Table 5). However, they did exhibit a percent flower weight more than 20%. This attribute is desirable to growers because plants with a greater ratio of flower to stem and foliage are easier to harvest (Darby H, Extension Professor, University of Vermont, Burlington, VT, USA, personal communication). The third study compared 2x and 3x genotypes for the same parental cultivar cross and found few differences between 2x and 3x counterparts (Tables 6 and 7, Fig. 4). Crawford et al. (2021) also found no differences in the cannabinoid content between 2x and 3x counterparts of 'TS1–3' × 'P163'. Fernandes et al. (2023) reported a cultivar-dependent response to polyploidization for the cannabinoid concentration of four cultivars. In 2022, 3x genotypes 'Tsunami' (4x) × 'Wife' (2x) and 'Wife' (4x) × 'Purple Star' (2x) were included in field trials at the University of Vermont (Burlington, VT, USA). Plants performed equivalent to or better than 14 industry-standard 2x cultivars and six 3x cultivars developed by Oregon CBD (Independence, OR, USA) (Darby 2023). Plants of 3x 'Tsunami' (4x) × 'Wife' (2x) exhibited the greatest percent flower weight at 46.6%. We found that greenhouse-grown 3x plants of 'Tsunami' (4x) × 'Wife' (2x) had a lower percent flower weight than their 2x counterparts, less total shoots, and smaller shoot length (Table 6). These findings suggest that differences in plant growth and flower production between 2x and 3x cannabis are most likely attributable to genotype, not ploidy. Furthermore, parental combination can influence important traits like cannabinoid content and percent flower weight. Several of our 2x and 3x genotypes were derived from photoperiod-insensitive and photoperiod-sensitive parents, or from parents with divergent backgrounds; therefore, enhanced performance observed for these genotypes may be partially attributable to heterosis (Bosca 1999; Kurtz et al. 2023; Small 2015).

In conclusion, 3x plants demonstrated reduced seed production but similar plant growth and flower production compared with 2x plants. Although 3x plants may develop a small number of filled seeds in the presence of pollen, there is low risk of large crop losses caused by seed set. The optimal cross direction for producing 3x seed is 4x × 2x because the 2x × 4x cross exhibits triploid block. When developing 4x plants, cryptic chimeral mixoploids can occur. If these are used as parents in the 4x × 2x cross, then the progeny will be a mix of 2x and 3x. For photoperiod-sensitive cultivars, a prolonged period of testing can identify cryptic chimeral mixoploids, but this is not possible for photoperiod-insensitive

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cultivars. For photoperiod-insensitive cultivars, it is necessary to produce 4x parents from seed by crossing two putative colchicine induced 4x plants. This method is preferred for photoperiod-sensitive plants and to ensure the 4x × 2x cross will yield all 3x seed.

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