Doubled-haploid Broccoli Production Using Anther Culture: Effect of Anther Source and Seed Set Characteristics of Derived Lines

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ADDITIONAL INDEX WORDS. *Brassica oleracea*, Italica Group, cole crops, anther culture, microspore culture, doubled-haploids, DNA flow cytometry, cabbage, cauliflower, Brussels sprouts

ABSTRACT. Using anther culture to generate doubled-haploid (DH) homozygous lines for use as parents in F₁ hybrid crosses has become a common practice in breeding broccoli (*Brassica oleracea* L. Italica Group). During anther culture and subsequent embryogenesis and plant regeneration, polyploidization of microspore-derived embryos may not occur or it may occur accompanied by a doubling, tripling, quadrupling, octupling, or irregular polyploidization of the genome. Thus regenerants from the process can be haploids, diploids, triploids, tetraploids, octaploids, or aneuploids. The objectives of this research were to 1) conduct repeat cycles of broccoli anther culture using a group of F₁ hybrids as anther donors and develop populations of regenerants; 2) analyze resulting populations using DNA flow cytometry and determine the influence of F₁ source on frequency of different ploidy levels among regenerants; and 3) compare seed set in broccoli inbreds developed in a traditional selfing program compared to seed set in DH broccoli derived from anther culture. In two cycles (1994 and 1995) of anther culture, anther-derived populations of regenerants were developed using the F₁ hybrids 'Marathon', 'Everest', 'High Sierra', and 'Futura' as sources of anthers. In 1994, 'Everest', 'High Sierra', and 'Futura' yielded populations that included 2% to 7% haploids, 53% to 56% diploids, 32% to 38% tetraploids, and 5% to 6% other types. 'Marathon'-derived regenerants were 5% haploid, 78% diploid, 15% tetraploid, and 2% other, showing significantly more diploids. In 1995, 'Marathon' regenerants again included significantly more diploids and fewer tetraploids than those derived from other F₁ sources, confirming that the genotype of the anther source affects the frequency of a particular ploidy level among regenerants derived from culture. In manual self-pollinations of 1994 regenerants, only diploids and rare tetraploids set seed. When plants that set no seed were discounted, seed production following manual self pollinations of 1995 regenerants was not significantly different from that of traditional inbreds derived from the same F₁ sources.

Broccoli (*Brassica oleracea*) cultivars in use today are almost exclusively F₁ hybrids. The most time-consuming and labor-intensive aspect of developing these hybrids is the traditional inbreeding process that requires manual self-pollination necessary to generate homozygous parental lines. This process requires six or more generations of inbreeding to establish adequately stable lines that can be used in hybrid combinations. Recently, broccoli breeders have increasingly used the regeneration of doubled-haploid (DH) lines from microspores via anther culture (Keller, 1984) or direct culture of microspores (Duijs et al., 1992) as alternatives to the slower inbreeding process. Production of *B. oleracea* plants from anther culture was first reported for cabbage (*B. oleracea* L. Capitata Group) by Kameya and Hinata (1970). In addition to work with broccoli (Keller and Armstrong, 1983), production of DH lines via anther culture has also been accomplished with Brussels sprouts (*B. oleracea* L. Gemmifera Group) and cauliflower (*B. oleracea* L. Botrytis Group) by Ockendon (1984 and 1988, respectively), with kale (*B. oleracea* L. Acephala Group) by Keller and Armstrong (1981), and with collard (*B. oleracea* L. Acephala Group) by M.W. Farnham (unpublished data).

A general problem in developing microspore-derived plants from *B. oleracea* crops using anther culture is that resulting populations contain a mixture of haploid, diploid, triploid, tetraploid, octaploid,

and aneuploid individuals (Chauvin et al., 1993; Chiang et al., 1985; Ockendon, 1988). These mixtures occur due to spontaneous and irregular polyploidization of microspore-derived embryos during the culture process. As a consequence, a breeder must specifically identify diploids among the mixtures of regenerants because these are the DH individuals that will serve as potential homozygous lines to be used in hybrid combinations. Individuals with any other ploidy level have little practical value and will divert the efforts of manual pollinators away from the true DH plants.

Ploidy of anther culture-derived cabbage was determined by chromosome numbers in pollen mother cells, and regenerants included 12% haploids, 55% diploids, and 33% tetraploids (Chiang et al., 1985). Guard cell length in the leaf epidermis was positively correlated with ploidy level to assess indirectly ploidy of anther-derived Brussels sprouts populations (Ockendon, 1986). In that case, ≈50% of regenerants was haploid and a similar percentage was diploid, while <5% had a greater ploidy level. Using leaf epidermis guard cell length to evaluate cauliflower regenerants, Ockendon (1988) found that 1% was haploid, 41% diploid, 5% triploid, and 53% tetraploid. Similar numbers for cauliflower were confirmed by Boucault et al. (1991) and Chauvin et al. (1993). Chauvin et al. (1993) were first to report the use of DNA flow cytometry for distinguishing ploidy of anther-derived regenerants of *B. oleracea* and found that, like cauliflower, anther-derived broccoli populations included about equal numbers of diploids and tetraploids and only a few individuals of other ploidy levels. Chauvin et al. (1993) observed poor seed production in DH broccoli and concluded that this represented a severe limitation to the value of DH plants as potential parents for making F₁ hybrids.

Chiang et al. (1985), Ockendon (1986), and Chauvin et al. (1993) all proposed that anther donor genotype in *B. oleracea* likely affects

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the frequency of haploids, diploids, tetraploids, and other ploidy levels in populations regenerated from anther culture. However, these researchers did not succinctly prove or disprove this hypothesis. Thus, the objectives of this work were to 1) conduct repeat (two) cycles of broccoli anther culture using a group of F_1 hybrids as anther donors and develop populations of regenerants; 2) analyze resulting populations using DNA flow cytometry and determine the effect of F_1 source on frequency of different ploidy levels among regenerants; and 3) compare seed production in broccoli inbreds developed in a traditional selfing program and in DH broccoli developed using anther culture.

Materials and Methods

ANTHER CULTURE AND REGENERANT PRODUCTION. Broccoli embryos were generated from microspores using standard protocols of anther culture (Keller, 1984) at the U.S. Vegetable Laboratory in Charleston, S.C. All phases of anther culture, including growth of donor plants, anther staging and bud selection, bud surface sterilization and anther extraction, culture media preparation, anther incubation, and culture of embryos and plant regeneration, were conducted as described by Keller (1984) with noted exceptions. Two alterations of the anther culture medium from the standard protocols included the addition of 14% instead of 10% sucrose and the addition of $2 \text{ mg} \cdot \text{L}^{-1}$ silver nitrate (AgNO_3). F_1 hybrid plants that served as anther donors were grown in a GC-15 plant growth chamber (Environmental Growth Chambers, Chagrin Falls, Ohio) set with a 16-h photoperiod and a day/night temperature regime of $15/10^\circ\text{C}$. In Winter and Spring 1994, broccoli anther cultures were initiated using the F_1 hybrids 'Futura', 'Marathon', 'High Sierra', 'Viking', 'Green Valiant', and 'Everest' as anther donors. Results with all hybrids except 'Marathon' were very similar in 1994. Therefore, to maximize the number of regenerants from 'Futura', 'Marathon', 'High Sierra', and 'Everest', 'Green Valiant' and 'Viking' were not used as anther donors in 1995. Each year, six plants of each hybrid were sampled for anthers. The entire culture process from initiation of anther cultures to establishment of microspore-derived, regenerated plantlets took 4 to 5 months. During the fall, conditioned broccoli regenerants were potted and moved to a temperature-controlled greenhouse without supplemental light and grown to maturity during the winter. Regenerants were grown in 25.4-cm-diameter pots filled with a commercial potting mix (Metromix 360; Grace Sierra, Milpitas, Calif.). Each broccoli regenerant grown in the greenhouse could be traced to an individual embryo in culture, and no two regenerants descended from the same embryo.

DNA FLOW CYTOMETRY. Anther-derived broccoli regenerants were prepared for DNA flow cytometry analysis using the protocol described by Arumuganathan and Earle (1991). Plants were sampled at an 8- to 12-leaf stage by cutting a 18-mm-diameter disk from a fully expanded leaf near the top of the plant using a cork-boring tool. Each plant was sampled on two different dates to confirm results. Preparation of samples for DNA flow cytometry involved 1) slicing leaf samples with a razor blade in MgSO_4 buffer containing propidium iodide to release and stain intact nuclei; 2) filtering of homogenates; 3) centrifuging filtrates; and 4) resuspending and continued staining of pelleted nuclei. The relative DNA content of nuclei suspensions and, indirectly, the ploidy level of sampled plants was determined by measuring fluorescence of respective propidium iodide stained nuclei preparations using a flow cytometer (EPICS XL; Coulter Electronics, Hialeah, Fla.) with an argon-ion laser operating at a wavelength of 488 nm. With this instrument, nuclei are analyzed for fluorescence intensity and histograms are generated, wherein frequency (number of nuclei) of a particular intensity is plotted against intensity.

About 50 samples were assayed in a single DNA flow-cytometry

session. Each session included controls consisting of individual leaf samples from known broccoli haploids, diploids, and tetraploids and a standard preparation of chicken red blood cells (Pocono Rabbit Farm and Laboratory, Canadensis, Pa.) run alone and combined with a diploid control. The flow cytometer was adjusted at the start of each session but not at all during the rest of the run. From session to session, settings (e.g., photomultiplier tube voltage adjustment) on the flow cytometer were usually the same. Haploid and tetraploid controls were found initially in preliminary runs of DNA flow cytometry, wherein standard broccoli cultivars (diploids) were compared to regenerants of unknown ploidy. Individuals exhibiting half or twice the fluorescence of diploids were identified, and these putative haploids and tetraploids, respectively, had ploidy levels confirmed using cytological evaluation of pollen mother cells (Keller, 1984). Confirmed haploids, diploids, and tetraploids were maintained vegetatively during these studies for use in flow cytometry.

DNA histograms from the flow cytometer for samples were compared to those of the known controls, and characteristic haploid, diploid, and tetraploid fluorescence intensities were identified (Fig. 1). Unknowns were classified based on these comparisons. Analysis of populations showed that $\leq 6\%$ of all samples exhibited some relative fluorescence intensity not characteristic of haploids, diploids, or tetraploids. This minor group had diverse histograms and included putative aneuploids, chimeras, triploids, and octaploids. Thus, they were grouped as an "other" category for comparing populations from different F_1 sources.

Statistical comparisons between the anther-derived populations of regenerants resulting from different F_1 sources were made using SAS categorical data modeling (CATMOD) Procedures (SAS Institute, Cary, N.C.). CATMOD analyzes data that can be represented by a two-dimensional contingency table (Tables 1 and 2). The rows of the table correspond to populations (e.g., regenerants derived from a particular F_1) formed based on independent variables. The columns correspond to observed responses (i.e., frequencies) formed based on dependent variables (i.e., ploidy level). The frequencies in the table are assumed to follow a product multinomial distribution corresponding to a sampling design in which a simple random sample is taken for each population and the analysis of frequencies allows for differentiation between the populations sampled.

MANUAL POLLINATIONS AND SEED PRODUCTION. In Winter 1994–95, bud self pollinations, as described by Williams (1985), were performed on all regenerants produced in 1994. Pollinations of each regenerant were repeated on at least two separate occasions, and each time 8 to 12 buds were pollinated. If a regenerant produced even one seed it was classified as a seed producer. In Winter 1995–96, only regenerants identified by flow cytometry as diploid (true DHs) were manually pollinated. Bud self-pollinations were again made on at least two separate dates with ≈ 8 to 12 buds pollinated each time. The exact number of buds pollinated at one time was recorded on pollination tags. When pods dried and seed matured (45 to 60 d after pollination), seeds were threshed by hand and counted and the number of seeds was recorded. For a given DH, the number of seeds produced per pollinated bud was determined. If a DH failed to produce seed it was considered a nonproducer and not used to estimate seed production of DH plants.

DH lines produced in 1994 were evaluated in a Fall 1995 field nursery. Eight to ten of these DH lines, which were developed from each of the hybrids 'Everest', 'High Sierra', 'Marathon', and 'Futura', were identified as the best DH lines from their respective populations with regard to desirable horticultural plant type and broccoli head quality. A plant from each of the best DH lines selected was grown and pollinated in the greenhouse during Winter 1995–96 with DH regenerants developed in 1995. Seed production on the best DHs was evaluated as described above.

F₄ broccoli plants from a pedigree selection program were grown during Winter 1995–96 in the same greenhouse as the confirmed diploids arising from anther culture. These F₄ plants originated from segregating populations developed by selfing 'Marathon', 'Everest', 'High Sierra', and 'Futura'. The individual F₄ plants were advanced from single F₂ and F₃ selections grown in Charleston field nurseries in Fall 1993 and 1994, respectively. All F₂ and F₃ selections from which F₄s descended were chosen as the best individuals, with regard to horticultural characteristics and particularly broccoli head quality, among all individuals in their respective segregating families. The F₄ plants were managed identically as DHs in the same greenhouse. Bud self-pollinations, seed collection, and measurement of seed production were made on the F₄s as described for DHs. Ten to twelve individual F₄ plants from each F₁ hybrid were pollinated and evaluated for seed production. As with the DHs, if an F₄ plant did not produce seed, it was not used to evaluate seed production. About 4% of the F₄s failed to set seed.

Statistical comparisons were made between DH and F₄ plants derived from the same F₁ source by conducting *t* tests of the seed yield means of respective DH and F₄ samples and assuming independent samples and equal variances (Steel and Torrie, 1980). These *t* tests used a weighted average of the sample variances.

Results and Discussion

DNA histograms from the flow cytometer for a control sample containing equal amounts of leaf material from haploid, diploid, and tetraploid individuals (Fig. 1A) and for four representative individuals, including a haploid (Fig. 1B), diploid (Fig. 1C), tetraploid (Fig. 1D), and a putative triploid (Fig. 1E), are illustrated. Profiles of analyzed individuals show two peaks of fluorescence intensity: one primary peak that corresponds to G₀+G₁ phase cells and a secondary peak that corresponds to G₂+M phase cells. Mean fluorescence intensity increases linearly with ploidy level, falling at ≈90 units for the haploid (C) peak, 180 for the diploid (2C), 360 for the tetraploid (4C), and 270 for the putative triploid peak (3C). Ninety-eight percent of duplicates from individual plants resulted in the same ploidy determination. Some of the remaining 2% of duplicate samples that exhibited different ploidy levels came from plants later determined to be ploidy chimeras, ploidy types also observed by Chauvin et al. (1993). Considering the existence of such chimeras, a 98% accuracy rate in typing ploidy of regenerants is an effective tool for identifying DH plants regenerated from anther culture among breeding populations commonly numbering in the hundreds and even thousands.

Except for 'Marathon', anther-derived populations from all F₁ sources cultured in 1994 had similar percentages of regenerants in all ploidy classes and were not significantly different from one another (Table 1). These populations of individuals were 2% to 7% haploid, 53% to 56% diploid, 32% to 40% tetraploid, and 3% to 6% other. These percentages are very similar to those observed by Boucault et al. (1991) and Ockendon (1986) in evaluations of anther-derived regenerants of cauliflower from single hybrid sources. Additionally, these populations have similar makeup as anther-derived populations from individual cauliflower and broccoli cultivars analyzed using flow cytometry (Chauvin et al., 1993). Categorical data modeling showed that the population derived from 'Marathon' was significantly ($P < 0.05$) different from all others. This population's makeup was 5% haploid, 78% diploid, 15% tetraploid, and 2% other. The more frequent occurrence of diploids in the 'Marathon' population was accompanied by a complementary reduction in the number of tetraploids.

In 1995, populations regenerated from anther culture using the

Fig. 1. DNA histograms from the flow cytometer of individual samples or regenerants. Each sample is represented by a histogram, which is a plot of cell counts (Y-axis) against fluorescence intensity (X-axis). Samples represented in histograms include a control sample (A) derived from equal amounts of leaf material from a known haploid, diploid, and tetraploid, and representative histograms of a haploid (B), diploid (C), tetraploid (D), and a putative triploid (E). The C, 2C, 4C, and 8C peaks are specifically indicated in A. Horizontal bars on each graph indicate ranges in positions of the C, 2C, 4C, and 8C peaks from left to right, respectively.

Table 1. Percentage of anther-derived broccoli developed in 1994 from different F₁ hybrid sources that have haploid, diploid, tetraploid or other ploidy level as assessed by DNA flow cytometry.

F ₁	Regenerants	Regenerant ploidy			
source	(no.)	Haploid	Diploid	Tetraploid	Other ^z
Regenerants (%)					
Marathon ^y	107	5	78	15	2
High Sierra	107	2	56	36	6
Futura	124	7	56	32	5
Everest	32	3	53	38	6
GreenValiant	55	2	55	40	3
Viking	31	3	55	39	3

^z"Other" includes putative triploids, octaploids, aneuploids, and ploidy chimeras.

^yThe population originating from 'Marathon' had a significantly different ($p < 0.05$) distribution than all other populations. The difference was determined using categorical data modeling procedures.

same hybrid sources as those used in 1994 were different than those from the previous year. Generally, in 1995 the frequency of tetraploids increased while the frequency of diploids decreased (Table 2). It is difficult to explain the differences between 1994 and 1995 since anther culture practices were the same; however, similar differential results between years were observed by Chauvin et al. (1993) for a single cultivar of cauliflower and by Ockendon (1986) for a single cultivar of Brussels sprouts. These observations of year-to-year or cycle-to-cycle differences emphasize the importance of comparing populations within a cycle and not between cycles. Although the two culture cycles in this study were different, the relative comparisons made in 1994 among populations derived from different F₁ hybrids were similar to those of the 1995 populations. As observed the year earlier, 1995 populations from 'High Sierra', 'Futura', and 'Everest' were not significantly different from one another and were 1% to 4% haploid, 32% to 44% diploid, 51% to 62% tetraploid, and 2% to 5% other. Conversely, the 'Marathon' population of regenerants was 4% haploid, 68% diploid, 26% tetraploid, and 2% other. This latter population was significantly different than all others and again contained a greater number of diploids with a complementary reduction in the tetraploid class. In total, results from the two cycles of anther culture provide evidence supporting the proposed hypothesis of previous researchers (Chiang et al., 1985; Chauvin et al., 1993; Ockendon, 1986) that genotype of the anther source can impact the frequency of diploids and other ploidy levels among individuals regenerated from culture.

Of all 1994 regenerants pollinated, only diploids and rare tetraploids set seed. No seed set was observed among pollinated individuals that were designated as haploids or putative aneuploids, triploids, or octaploids. Of 138 pollinated tetraploids, only three set seed, and only one or two seed were produced by each of these. Overall, 25% to 43% of all regenerants pollinated, depending on F₁ source, set seed on selfing (Table 3). Among 1994

pollinated diploids, ~50% produced seed regardless of the original F₁ anther source (Table 3). Chiang et al. (1989) observed ~50% sterility among a sample of cabbage DHs regenerated from anther culture. In these same DHs, Chiang et al. (1989) observed meiotic abnormalities in microspore mother cells, including a high occurrence of laggards at anaphase I and telophase I and unequal chromosome segregation and micronuclei at anaphase II and telophase II. These abnormalities partially explained sterility among the DH plants. However, these authors also concluded that many sterile individuals likely occur due to the severe inbreeding that has taken place in going to complete homozygosity. Because nonfertile diploid regenerants have no potential value as DH parental lines, their frequent occurrence in DH populations significantly decreases the efficiency of anther culture for line development.

When only diploids were pollinated among the regenerants produced from culture in 1995, 64% to 75% of confirmed DHs set seed (Table 3). This represented an increase in the percentage of fertile DH plants over 1994 for all populations evaluated. The nonproducing DH plants again had no potential as DH lines and could not be evaluated as seed producers. By identifying DHs among regenerants and focusing pollination efforts on them, as was done with 1995 populations, effective pollinations were increased dramatically compared to 1994 when all regenerants were pollinated. Because manual self-pollinations represent a major cost in breeding broccoli, using DNA flow cytometry to confirm that a regenerant is a diploid before pollinating it could save considerable time and effort, making it a cost-effective practice.

Evaluating seed production in the greenhouse during 1995–96, I was unable to demonstrate a statistical difference between DHs derived from anther culture and F₄ DHs derived from anther culture and F₄ individuals developed through traditional pedigree selection and inbreeding when nonseed-producing plants were eliminated from consideration

Table 2. Percentage of anther-derived broccoli developed in 1995 from different F₁ hybrid sources with haploid, diploid, tetraploid, or other ploidy levels as assessed by DNA flow cytometry.

F ₁	Regenerants	Regenerant ploidy			
source	(no.)	Haploid	Diploid	Tetraploid	Other ^z
Regenerants (%)					
Marathon ^y	121	4	68	26	2
High Sierra	180	3	44	51	2
Futura	98	1	34	60	5
Everest	74	2	32	62	4

^z"Other" includes putative triploids, octaploids, aneuploids, and ploidy chimeras.

^yThe population originating from 'Marathon' had a significantly different ($p < 0.05$) distribution than all other populations. The difference was determined using categorical data modeling procedures.

Table 3. Percent of all anther-derived broccoli from 1994 and percent of all doubled haploids (DHs) in 1994 and 1995 to produce seed following self pollination. Each population of regenerants was developed using a different F₁ source.

F ₁ source	Regenerants to set seed (%)	DHs to set seed (%)	
	1994	1994	1995
Futura	29	51	75
High Sierra	30	53	64
Marathon	43	55	64
Everest	28	53	75
Green Valiant ²	25	46	---
Viking ²	28	50	---

²Anther-derived populations from 'Green Valiant' and 'Viking' were not developed in 1995.

(Table 4). Mean seed production ranged from 2.4 to 5.8 seed per pollination for the F₄ plants from the four F₁ sources, from 1.8 to 3.5 for all DHs, and from 2.0 to 4.4 for the phenotypically best DHs. In all cases, the mean number of seed per pollination was numerically higher for F₄s compared to all DHs. Additionally, mean numbers of seed per pollination for the best DHs approached the means for F₄ seed production. However, *t* tests identified no statistically significant differences. Chauvin et al. (1993) observed seed set in selfed DH broccoli plants and concluded that seed production of DHs is inadequate and a likely limitation to perpetuating and using them as possible parents of hybrids. Present results do not confirm this conclusion. In fact, my results indicate that, if a breeder focuses on DH individuals capable of setting seed, he should find that seed production will probably not be a limiting factor in using those plants. More importantly, it appears that when one selects the best broccoli phenotypes among DH lines of a given population, one may indirectly be selecting for better seed producers. I have observed some selected high-quality DH lines that are prolific and consistent seed producers, typically yielding more seed on selfing than most inbreds, regardless of how they were developed.

The ultimate test of a broccoli DH line as a parent of a hybrid is how well it combines with another inbred to make a high-quality and productive F₁. However, before undergoing such tests for combining ability, a DH line must have good phenotypic value per se and be readily perpetuated as an inbred line. Results of this work indicate that, once good-quality phenotypes in a DH line are identified, perpetuation of that line by seed should not be a limitation to its further use. The fact that several seed companies now market F₁ broccoli hybrids developed using at least one DH line derived from anther culture indicates that limitations of the process have not prevented its use to produce commercial parental lines.

'Marathon' is a specific genotype that produces a relatively high frequency of DHs from anther culture, with 78% DHs in 1994 and

68% in 1995. These DH percentages of anther-derived populations are higher than previously reported for *B. oleracea*. It is fortuitous that 'Marathon' is the number-one selling broccoli hybrid in the United States (Sakata Seed Co., personal communication) and that many DH lines derived from it have good phenotypes. In the future, one might identify a DH line derived from 'Marathon' that has good combining ability and that when crossed to other inbreds results in F₁s that give a high frequency of DHs when anther cultured. Identification of such an inbred would increase the efficiency of broccoli anther culture for producing useful DH lines.

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Table 4. Means and standard errors (in parenthesis) for seed production following self pollination of F₄ plants developed via traditional inbreeding, seed-bearing doubled haploids (DHs) developed in 1995, and plants from the best horticultural DH lines observed in Fall 1995 in the field. F₄ and DH plants were all developed starting with the same F₁ hybrids.

F ₁ source	Seeds/pollination		
	F ₄ plants	All DH plants	Best DH plants ²
Futura	2.4 (0.6)	2.0 (0.4)	2.0 (0.7)
High Sierra	3.3 (0.7)	1.8 (0.3)	2.8 (0.7)
Marathon	3.1 (0.8)	2.0 (0.4)	2.9 (0.7)
Everest	5.8 (1.5)	3.5 (0.9)	4.4 (0.8)

²The best DH plants are a group (eight) of plants representing doubled-haploid lines that exhibited the best horticultural phenotypes compared to all other DH lines in their respective DH populations that were evaluated in a Fall 1995 field nursery.