

Development of Improved Spice Crops
using Double Haploid Technology

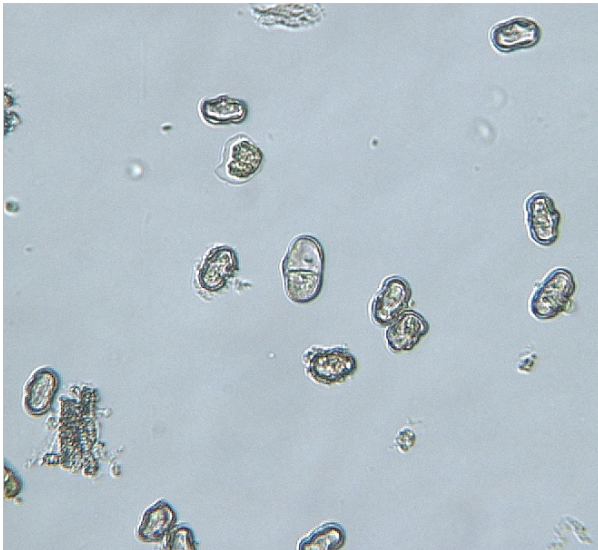
(Feb 2009 Final Report)

ADF Project No. 20050711

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Abstract

NRC/PBI has used double haploid (DH) technology to create new lines of a range of spice crops (dill, fennel, anise, caraway) of interest in Saskatchewan. *One objective of this project was to evaluate these DH lines in replicated field trials.* DH dill and caraway lines were evaluated in 2006 through 2008. Several of the DH dill lines had agronomic characteristics like short uniform stature along with early and concentrated seed maturity that could be useful for crop improvement. Seed yields and seed oil content of the most adapted DH dill lines were either equal to or higher than the parental lines. The essential oil composition (flavor/odor) of the DH dill lines was comparable to the parental lines. This project also identified a DH line of annual caraway that produced substantially higher seed yields than the line presently regarded as the industry standard.

Cumin has been identified as an opportunity crop for Saskatchewan, however grower success with cumin has been limited due to a lack of lines adapted to Saskatchewan growing conditions and production practices. *The second objective of this project was to generate variation in cumin through mutagenesis and/or double haploidy, with the objective of creating lines of cumin better suited to Saskatchewan growing conditions.*

- a. Cumin microspore culture is the first step in generating double haploid plants. A number of factors influencing microspore embryogenesis in cumin were evaluated – donor genotype, donor plant growing conditions, culture medium and culture conditions. Some microspore swelling and divisions occurred, but despite manipulation of over 900 different experimental variables, no further development was observed.
- b. Seed mutagenesis experiments were conducted using EMS (ethyl-methane sulphonate) as the mutagen. Germination of the treated cumin seeds decreased as exposure to the mutagen increased, however, even at the maximum tolerable dosage of EMS, there were no morphological differences in the plants derived from the mutagenized seed.

Introduction and Project Rationale

Spice crop production represents an opportunity for diversification and value-added processing in Western Canada. However, despite favorable prices and market opportunities, production of many spice crops has failed to expand on the Prairies. This reflects a lack of lines adapted to local climatic conditions and pest pressure. If this limitation can be overcome, there is significant potential for expanded production and processing of these crops in Saskatchewan.

Genetic variation is essential for plant breeding/improvement programs. Previous screening of cumin germplasm for adaptation to Saskatchewan's growing conditions and production practices has failed to identify any planting material suited to Prairie conditions (ADF Project No 2002-0163 - Waterer and Wahab). Therefore, other methods to generate variation need to be employed. Conventional mutagenesis e.g. seed mutagenesis, of plants has been used in both ornamental and crop plants to increase variation and improve yield, quality, disease and pest resistance and flower colour and morphology. More than 1700 mutant varieties have been released from mutation breeding programs. This includes both vegetatively and sexually propagated species.

This project proposed to institute a mutation breeding program under the direction of Dr. Ferrie, with the objective of working on several of the key characteristics needed in cumin ie; increased

seedling vigor, great height, better stem strength, and enhanced disease resistance. The starting material for this mutation breeding program was cumin lines previously identified by Dr. Waterer and the industry as having the greatest promise for production in Saskatchewan.

Double haploid technology represents another effective method for both identifying promising material for breeding programs and for speeding the development of homozygous lines suited for market release. This is beneficial to the producer, as high yielding cultivars with disease resistance and improved seed quality can be developed more rapidly. Having a product that is consistently of high quality is also beneficial to the consumer and will be important from the regulatory standpoint.

In preliminary field trials evaluating the double haploid lines of dill already generated by PBI/NRC we discovered DH lines that were substantially more uniform in height and maturity than the parent lines with greater seed yields. We also identified lines with either a higher seed oil content or an improved oil quality profile relative to the parental material. In corresponding trials with annual caraway we identified lines with enhanced seedling vigor and superior yield potential relative to the parental lines. The extent and speed of improvement achieved with this double haploid program far exceeded the progress that could be realistically expected within the same time period from a standard breeding program. A great deal of the material generated by the PBI/NRC double haploid program has yet to be evaluated and consequently its potential remains unutilized.

This field trial component of this project (co-ordinated by D. Waterer) was designed to more fully evaluate the double haploid lines of spice crops that have already been generated by PBI/NRC and was slated to also field test any new lines of cumin generated by PBI/NRC as a function of this project.

Project Progress to Project Completion (Feb 2009).

Objective 1. Field Testing of DH lines

2006 - DH Dill trials

In 2006, the main testing trial utilized the DH lines of dill generated by PBI/NRC that had shown the most promise in small scale screening trials conducted in 2004 and 2005. Lines were selected and multiplied for further testing if they showed promise in any of the following areas;

- a) plant vigor, uniformity of height, uniformity of maturity
- b) seed yield
- c) oil content of the seed
- d) chemical composition of the seed oil.

Key objectives were to identify lines that retained the seedling vigor of the parental lines, but were shorter and more uniform in stature. Improved uniformity of seed set was a highly desirable characteristic. The presently available commercial dill lines have a very indeterminate growth habit resulting in uneven seed maturity. With highly indeterminate seed set it is inevitable that a portion of the seed is lost to shattering before the crop is ready to harvest ... while a portion of the seed is also still immature at harvest. Immature dill seed does not have the flavor profile required by the industry.

All field trials were conducted at the University of Saskatchewan Horticulture Research Facility in Saskatoon (2909-14th Street East). This field site features a Sutherland Series heavy clay soil. This soil type is less than ideal for establishment of small seeded crops - but once the crops get established, its moisture and nutrient retention characteristics result in excellent yields. The test site had been in summer fallow in 2005.

The field was rotovated prior to planting to provide as uniform a seed bed as possible. No supplemental fertilizers were required. The trial was seeded on May 23. This is a later planting date than most commercial growers would use - but it reflects the fact that the clay soil at the test site is very slow to dry and warm in the spring. The crop was seeded with a push-type small plot seeder. Each line was planted out in 3 rows (0.5 m apart) with each row being 8 m long. Each plot was separated by a 1 m buffer. The trial was laid out in a randomized complete block design with 3 replicates.

Most of the seed used in the 2006 trials was generated in 2004 and 2005 in small plot observation trials conducted by the U of S. Because of adverse growing conditions in 2004 and problems with herbicide injury in 2005, the viability of the seed was relatively low and also varied between lines. This was confirmed in germination tests conducted prior to seeding the 2006 crop (Table 2006-1). To compensate for the low seed viability, we opted to use an exceptionally high seeding rate - roughly 4X normal. This resulted in a very dense stand in the lines with the highest germination % , but only a spotty stand in the lines with the poorest germination % . Like canola, dill is an indeterminate crop. Dill plants are capable of altering their branching habit to fully utilize the available growing space. Consequently, once the plants reached maturity, the plots for the various lines were relatively uniform in terms of canopy density.

Warm weather coupled with timely rainfall in early June resulted in rapid and uniform crop emergence but also produced near-ideal conditions for the germination of weeds. The herbicide linuron (Lorox) was applied (200 g a.i./a) once the crop reached the 3 true leaf stage. The herbicide was lightly watered in 4 days after application. Linuron is cleared for use on a wide range of Umbelliferous crops (ie; coriander and carrot) - but it is not presently registered for use on dill. Rick Holm (weed scientist at the U of S) has previously tested linuron on dill and found it to be quite crop safe. However, when this product was used on the 2005 seed increase of the DH dill, it caused significant crop damage (yellowing and some seedling death). This problem was initially attributed to adverse conditions following application of the herbicide. However, similar problems occurred again in 2006 - and in that case conditions at and following application of the linuron were near-ideal. The degree of damage was similar in both the parental lines and the double haploids. Relatively few plants actually died as a result of the herbicide treatment in 2006, but growth of the crop was set back by at least 3 weeks. The linuron provided excellent season-long weed control in this trial.

In a presentation made to the 2006 Annual Meeting of the Canadian Society for Agronomy, Zheljzakov *et al* showed that while application of linuron to dill caused about 30% plant mortality, **this degree of damage had**



Fig. 2006-1. Impact of herbicides on vigor of DH dill. Line on right has not been sprayed.

no impact on yields. This illustrates the yield plasticity of this crop. They identified a number of herbicides that were less toxic to the crop than linuron - these products should be considered for use in future work with dill.

By early July, the dill crop had out-grown the effects of the herbicide – except that the sprayed plants were considerably shorter than plants in areas missed by the spray. This is illustrated in Figure 2006-1. The crop was irrigated once in late July.

By the 3rd week in August, the flowers and some of the upper leaves on some of the lines had begun to yellow. Initially this was attributed to these lines being early maturing - but it turned out that the yellowing was largely due to disease. The disease started in a couple of localized points, but spread throughout the plot with a one week period. Although the causal organism was not isolated, the symptoms are typical of blossom blight. This disease complex has become fairly widespread in commercial plantings of coriander and caraway in Saskatchewan - but dill is usually not as severely affected. Distribution of the disease was not uniform across the plots - but whether this reflects differences in disease sensitivity of the various DH lines - or was due to differences in distribution on the initial disease inoculum could not be determined. Because of the rapid spread of the disease, no chemical control was attempted. Trials conducted by the University of Saskatchewan with blossom blight of coriander suggest that this disease cannot be controlled utilizing commercially available foliar-applied fungicides once it has become established within the canopy. Fungicidal seed treatments followed by a preventative spray program may be more effective - and this practice was followed in subsequent trials.

Each line was straight cut combined as it matured using a WinterSteiger Plot Master combine. Due to the indeterminate nature of dill, some pre-harvest shattering loss of the first seed set is inevitable. In 2006, the onset of wet, cool weather in mid-September resulted in a substantial delay in the harvest of many of the later maturing lines (September 26). Unfortunately, once dill is ready to the crop is ready to harvest, anything delay can result in substantial losses to shattering. Although swathing may reduce problems with shattering, most commercial growers try to avoid swathing dill because of its rank growth habit. It may be useful to try to chemically desiccate the later maturing lines.

The seed was air dried after harvest and then cleaned using a dockage tester.

Table 2006-1. Agronomic Observations on Double Haploid and Parental lines of Dill (2006).

Line	Germ. % ^a	Flowering % ^b	Height (cm) and cv (%) ^c	Shattering (0-5) ^d	Yield (kg/ha)	% grade out
DH-1	5	25% d ^e	104 c (21%)	Minimal stand	59 e	55 ab
DH-7	43	12% ef	119 ab (13%)	1.7	640 ab	47 ab
DH-12	35	100% a	72 f (13%)	Harvested Aug 20th	142 de	60 b
DH-35	33	15% de	118 b (11%)	2.7	485 bc	56 ab
DH-45	36	47% c	97 d (14%)	2.6	339 cd	55 ab
DH-47	75	10% ef	83 e (11%)	2.3 + severe blight	59 e	76 c
DH-53	20	3% f	127 a (8%)	3.7	774 a	45 a
Mammoth	15	78% b	109 c (14%)	1.7	352 cd	46 ab
CDC Giant	75	88% b	92 d (15%)	Severe blight	170 de	58 ab

^a Germination % in petri dish assays

^b Flowering rated July 20th

^c Height measured Aug 26th.

^d Shattering severity rated on Sept 28. Ratings .. 0= none, 5 = 100%

^e Values within columns followed by the same letter are not significantly different ($p=0.05$)

Yields per unit area were below the average of 1000 kg/ha seen in most commercial dill fields - this likely reflects the fact that the rows were very widely spaced to facilitate roguing and crop evaluation. Of more importance is the fact that several of the DH lines had yields that were substantially greater than the industry standards (Mammoth and Giant). It is noteworthy that the yields exceeded that of Mammoth – as it was Mammoth that was used to generate the DH lines included in this test.

Oil content and quality were evaluated utilizing standard methods (Arganosa et al 1999). Briefly, a 5 g sample of cleaned seed was steam distilled to extract the essential oils and then the composition of the oil is determined by HPLC.

The results of the oil analysis and the corresponding oil yields are presented in Table 2006-2. Overall, the oil content was slightly higher than normal – this likely reflects the warm growing conditions through July and August of 2006. Although there was a significant degree of variation in the essential oil content of the various lines tested, the composition of the oil (limonene : carvone ratio) was quite similar (Table 2006-2).

Table 2006-2. Oil content and composition for double haploid and parental lines of dill (2006).

Line	Essential oil (%)	Limonene (%)	Carvone (%)	Yield (kg/ha)	Oil yield (kg/ha)
DH-1	2.7	51	40	59	1.6
DH-7	2.7	53	40	640	17.3
DH-12	3.3	50	44	142	4.7
DH-35	2.4	50	43	485	11.6
DH-45	2.2	48	46	339	7.6
DH-47	2.2	52	41	59	1.3
DH-53	3.0	50	46	774	23.2
CDC Giant	2.9	50	45	170	4.9
Mammoth	3.1	50	45	352	10.9
Average	2.7	50.5	43.7	335	9.2

The essential oil content of DH 12 was superior to all other DH lines, as well as the industry standard lines.

Notable performance, yields and quality characteristics of the lines were;

- a. Line DH-12 had performed very well in previous trials – but in this trial it appeared to experience a greater degree of damage by the herbicide than the other lines. This may reflect the fact that DH-12 emerges very quickly – resulting in this line being at a more advanced stage of crop development at the time of the herbicide was applied. This may have rendered it more susceptible to herbicide damage. DH-12 is also very uniform in height, with a short stature and early maturity (Table 2006-1). Figure 2006-3 shows that on July 20, DH-12 was in full bloom – whereas most of the other lines were just starting to bud out at that time. In the 2006 trial this meant that this line was flowering at a time when weather conditions appeared to be most suitable for development of the blossom blight. As a consequence many of the seeds formed by this line were shrunken and sterile. This is reflected in the exceptionally high grade out and low 1000 seed weight for DH-12. Yields for this line were consequently lower than normal. However, DH-12 is very early maturing. This is reflected in the exceptionally high oil content of seed of this line.
- b. Line DH-47 is another short stature line that had performed well in previous trials. Although this line showed excellent seedling vigor, it was quite severely affected by blight, resulting in low seed yields and a high % seed grade out due to shrunken (blight affected) seeds (Table 2006-1). The poor quality of the seed harvested for this line was also reflected by its low oil content (Table 2006-2).
- c. CDC Giant - experienced some of the same herbicide toxicity problems as D12. This line also appeared to be particularly sensitive to the blossom blight. The essential oil content of CDC Giant was quite high, reflecting the strong selection pressure for this trait in dill breeding programs.

- d. Lines DH-7 and DH-53 - in this trial, as in previous trials, these lines were very vigorous, producing plants that were taller than the parental line (cv Mammoth) but much more uniform in height. Both of these lines were relatively late maturing. Harvest of these lines was delayed due to bad weather from mid-September onwards. This resulted in extensive seed losses to shattering. Despite this loss, these lines were the highest yielding in the trial. In part, this could be attributed to the fact that these lines were relatively unaffected by the blight. Whether this reflects inherent disease resistance or simply reflects differences in growing conditions at the key stages of development of both the crop and the disease infection could not be determined. Although these lines were both quite late, the essential content of the seed was acceptable – with DH 53 having a seed oil content superior to CDC Giant. **Overall oil yield/unit area of Lines DH-7 and DH-53 were respectively 58% and 116% greater than the industry standard Mammoth – this reflects both the high yields and relatively high seed oil content of these lines.**
- e. Line DH-1 – in previous trials this line had been extremely late but had produced very large plants with very large seeds. Seed viability was very poor – likely reflecting problems with getting mature seed. The poor seed viability led to a poor stand and seed yields were consequently low. The seed oil content was also quite low.

Evaluation of New DH Dill Lines

Over the winter of 2005/2006 the program generated seed of the DH lines that had produced the best results in the 2004 and 2005 field trials (DH 12, 47 and 53). This step was considered prudent given the poor viability of the seed generated in the 2004 and 2005 field trials. This seed was used to establish a nursery for seed generation of these DH lines. Using transplants to establish the nursery insured that high quality mature seed could be harvested in a timely manner. It also allowed for evaluation of the germination %, field performance and seed quality of the selected lines against the parental lines. Two new DH lines were also included in this trial.

Line	Parent	% germination	Stature	Maturity
DH12	Mammoth	52	Short and uniform	Early and uniform
DH47	Mammoth	65	Moderate and uniform	Early and uniform
DH53	Hercules	42	Tall and uniform	Late
DH54	Fernleaf	0	-	-
DH55	Hercules	0	-	-
Parental	Hercules	67	Tall and uneven	
Parental	Mammoth	74	Very tall and uneven	Late, variable
Parental	Fernleaf	92	Very short	Very late

Results – The germination % of the previously selected DH lines was not as good as the parental types, but once in the field these DH lines again out-performed their corresponding parental lines, producing uniform stands of vigorous plants. DH 12 and 47 were far earlier to mature than the parental lines, while DH 53 produced a very vigorous, uniform stand of late maturing but high yielding plants. It is interesting to note that there was very little disease in this trial – while these same lines (DH 12 and DH 47) had been severely affected by disease in the adjacent direct seeded trial. This suggests that disease losses in this crop are strongly influenced by interactions between disease, crop development stage and environmental conditions. The two new DH lines included in this trial failed to germinate.

2006 – DH Caraway trials

A total of 25 DH lines of caraway were available for the 2006 trials – along with 3 parental lines. Because of limited amounts of seed available for the DH lines, all lines were seeded in the Dept of Plant Sciences greenhouses and then transplanted out in early June. The seedlings were raised in 144 cell transplant flats filled with Sunshine Mix #4 media. The flats were maintained under near optimum conditions for germination – a 24/18 C temperature regime with a 16h photoperiod. When the seedlings were 4 weeks old they were transplanted into the previously described field plot area (15 cm apart with 0.5 m between rows). The growth habit of the plants was observed. The trial was hand harvest in early October and then threshed using a stationary combine. The seed was cleaned using a dockage tester. Because of the uneven numbers of plants no yield comparisons were possible.

General observations

The parental material showed relatively poor seed viability even under the near-ideal conditions of the greenhouse (Table 2006-3). Once transplanted into the field, the parental lines performed relatively well – they appeared vigorous and were quite uniform in stature and maturity. No problems with disease were observed in the parental or the DH lines.

Table 2006-3. Germination %, seed oil content and seed oil composition for DH caraway lines tested in 2006

Line	Parent	% germination	% essential oil	Limonene %	Carvone %
DH6	NN-1	0			
DH6	Moran	0			
DH7	Moran	0			
DH8	Moran	0			
DH9	Moran	0			
DH10	NN-2	100	1.29	42.6	51.4
DH11	Moran	0			
DH12	Moran	0			
DH13	NN-2	0			
DH14	Moran	7	0.86	59.4	34.8
DH15	NN-2	1			
DH16	NN-1	0			
DH17	NN-1	6	1.36	48.8	47.0
DH18	NN-2	0			
DH19	NN-2	0			
DH20	Moran	7			
DH21	Moran	28	2.38	47.2	47.5
DH22	Moran	4			
DH23	NN-2	0			
DH24	NN-2	0			
DH25	NN-2	0			
DH26	NN-2	0			
DH27	NN-2	9			
DH28	NN-2	1	1.43	49.5	42.6
DH29	NN-2	78	0.69	51.1	31.8
Parental	NN-1	36	2.46	49.9	47.9
Parental	NN-2	36	1.42	42.7	51.5
Parental	Moran	20	1.51	48.5	49.3

Yellow highlights represent a significant improvement, red highlights represent a significant disadvantage and blue highlights represent a significant change relative to the parental line.

As has been seen in other crops, many of the DH caraway lines had serious agronomic drawbacks - ie; many had low germinate % even under ideal greenhouse conditions (Table 2006-3), or they had very poor vigor or an exceptionally short or distorted growth habit. Although some of these poorly adapted lines did produce seed, they will likely be omitted from future trials. Some of the DH lines (ie; DH-10 and 29) showed much better germination % than the corresponding parental lines. None of the DH caraway lines were obviously superior to the parental lines in terms of vigor, uniformity of plant configuration or yield.

Seed quality assessments were conducted on 5 g samples of clean seed, utilizing the procedures outlined in the dill section. The essential oil content of line DH-21 was significantly higher than the parental line, while for lines DH-14 and 29 the essential oil content of the seed was far lower than the parent. The oil composition of these lines was also quite different from the parent. The

combination of low oil content and different oil composition may reflect the fact that the seed of these lines was not fully mature at harvest.

Sufficient seed was generated in this trial to allow a replicated yield trial to be conducted in 2007.

2006 – DH Carrot trials

A total of 17 DH carrot lines were available for the 2006 trials – along with 1 parental line. Because of limited amounts of seed available for the DH lines, all lines were seeded in the Dept of Plant Sciences greenhouses and then transplanted out in early June. The seedlings were raised in 144 cell transplant flats filled with Sunshine Mix #4 media. The flats were maintained under near optimum conditions for germination – a 24/18 C temperature regime with a 16h photoperiod. When the seedlings were 3 weeks old they were transplanted into a field plot that was being used to evaluate a range of varieties of standard carrots. This field featured a series of raised beds (0.3 m high, 0.3 wide, with 1 m between beds). The growth habit of the plants was observed until the trial was hand harvest in early October.

General observations

As has been seen in the other crops tested, use of DH technology can have serious agronomic drawbacks – ie; **all DH carrot lines except for DH 17 failed to germinate under the near-ideal conditions in the greenhouse.** DH 17 had about 30% germination as compared to 67% germination for the parental line. If the DH seedlings survived through the first 4 weeks they usually went on to produce a healthy and vigorous plant. Unfortunately, growing carrot seedlings in confined spaces (ie; seedling trays) causes the tap roots to grow abnormally. Problems with root distortion were further exacerbated by problems at the time of transplanting - it was very difficult to orient the long, thin tap roots of the seedlings in a manner that allowed their subsequent growth to be “normal”. In most cases, the developing tap root failed to penetrate into the soil and consequently any subsequent growth pushed the root out of the soil. These exposed roots developed a distorted growth habit and took on an abnormal sunburnt appearance. This problem occurred in both the DH line 17 and in the parental type. Because of the abnormality of the crop’s growth habit, it was difficult to evaluate DH line 17 against the parental line. DH 17 appeared to be similar to the parental line in terms of vigor, uniformity of plant configuration or root size.

A greenhouse multiplication trial was conducted through the winter of 2006/2007 in an attempt to generate sufficient seed to allow this trial to be planted out using standard cropping practices in 2007. At harvest, the carrots were topped and then the roots were placed in moist peat moss in cold storage (2°C) for 6 weeks. This cold period is necessary to trigger flowering once the crowns are planted out. The crowns were planted out in the University of Saskatchewan greenhouses in December of 2006. Although most of the crowns began to regrow fresh tops it became apparent that the plants were severely infested with aster yellows. This mycoplasma was fairly widespread in the nearby carrot yield trials conducted in 2006. Leaving the DH plants in the field late into the fall in order to maximize pre-harvest cold treatment also appears to have exposed the plants to high levels of the leaf hoppers that serve as vectors for aster yellows. Growth of the aster yellows infected plants was weak with many abnormal leaves. Most plants died before flowering and those that did flower failed to produce any viable seed.

2007 - DH Dill Trials

The 2007 trial utilized the most promising DH lines selected from previous trials. Lines were included in these trials if they had demonstrated promise in any of the following areas;

- a) plant vigor, uniformity of height, uniformity of maturity
- b) seed yield
- c) oil content of the seed
- d) chemical composition of the seed oil.

Key objectives were to identify lines that retained the vigor of the parental lines, but were shorter and more uniform in stature. Improved uniformity of seed set was a highly desirable characteristic. The presently available commercial dill lines have a very indeterminate growth habit resulting in uneven seed maturity. With highly indeterminate seed set it is inevitable that a portion of the seed is lost to shattering before the crop is ready to harvest, while a portion of the seed is also still immature at harvest. Immature dill seed does not have the flavor profile required by the industry.

Unless otherwise specified, the procedures utilized in the 2007 DH dill trials corresponded to the procedures previously described for the 2006 trials. The trial was again conducted at the University of Saskatchewan Horticulture Research Facility in Saskatoon. The test site had been in pumpkins in 2006 which resulted in greater weed pressure than in previous years. The field was prepared as previously described. The trial was seeded on May 23. This is a later planting date than most commercial growers would use - but it reflects the fact that the clay soil at the test site is very slow to dry and warm in the spring. The crop was again seeded with a push-type small plot seeder. Each line was planted out in 3 rows (0.5 m apart) with each row being 8 m long. Each plot was separated by a 1 m buffer. The trial was laid out in a randomized complete block design with 4 replicates.

The seed used in the 2007 trials was generated in the 2006 trial. Although the germination % of the seed was relatively good, we still opted to seed relatively heavily because of the heavy nature of the soil at the test site. This resulted in a dense stand for most lines except DH 45 and 47 (Table 2007-1). By July, the crop canopy for the various DH dill lines appeared quite uniform – despite the difference in initial plant stand. This reflects dill's indeterminate growth habit.

Warm weather coupled with timely rainfall in early June resulted in rapid and uniform crop emergence but also produced near-ideal conditions for the germination of weeds – particularly red root pigweed and common groundsel. Weed pressure was particularly heavy in the 4th block of this trial. In the 2006 trial the herbicide linuron (Lorox) applied at 200 g a.i./a once the crop reached the 3 true leaf stage had caused significant crop damage (yellowing and some seedling death). In the 2007 trial we again opted to use Linuron, but at a lower rate (150 g a i./a). The lower rate of linuron reduced but did not eliminate herbicide damage to the crop (see Table 2007-1) and also provided less effective weed control – especially in the heavy weed pressure in the 4th block. The 4th block had to be hand weeded on several occasions in 2007.

In the 2006 trial, the DH lines as well as the industry standard and parental lines had appeared uniformly susceptible to the relatively high rate of linuron. In the 2007 trial when the lower linuron rate was used there appeared to be some variability in relative sensitivity – with several of the DH lines showing more damage than the established lines (Table 2007-1). Whether these differences reflect actual differences in herbicide sensitivity or are simply related to differences in crop developmental stage at the time of exposure to the linuron could not be determined. The

fact that CDC Mammoth seemed particularly resistant to linuron in 2007, yet in the 2006 trial it was amongst the most sensitive lines, suggests that the effect was mediated by environmental conditions or developmental stages.

By early July, the dill crop had out-grown the effects of the herbicide, but development and vigor of the 4th block was delayed by weed pressure. The crop was irrigated once in late June and again two weeks later. Although the soil was quite dry from mid-July onwards we opted to not irrigate because of the problems with blossom blight observed in the 2006 trial.

Because of the problems with blossom blight observed in 2006, we opted to implement a preventative spray program in 2007. The crop was sprayed with azoxystrobin (Quadris) or chlorthalonil (Bravo) every 10 days from mid-July as the crop began to come into flower through until mid-August by which time the crops had begun to mature. There were no obvious signs of blossom blight in 2007 – whether this indicates the spray program was effective could not be determined as there were no untreated areas for comparison. Although there was no disease apparent, some lines again flowered well yet set relatively few seeds and many of those seeds were shrunken and of low quality. Whether this problem reflects a disease or is a varietal response to adverse growing conditions at flowering could not be determined.

Seed loss due to shattering, both prior to harvest and during combining, is a major issue during the harvest of dill. In previous years the crop was straight cut combined once it had matured – but this resulted in significant shattering loss, particularly in early maturing lines or in situations where die-down was delayed by cold, wet weather. In 2007 we opted to chemically desiccate the crop with diquat (Reglone at 1.0 l/a) as soon as it began to mature, followed by hand swathes 5 days later to minimize shattering due to wind action. One week later the swathes were picked up and combined using a standard WinterSteiger small plot combine. Although this harvest procedure did not completely eliminate shattering loss, the losses were far lower than in previous years. This is reflected by the fact that **yields in 2007 were almost 10X higher than in 2006 (Table 2007-1)**. Although the altered harvesting procedures may have made a significant contribution towards the higher yields observed in 2007, near ideal growing conditions through September followed by very favorable harvest conditions were also helpful.

Yields per unit area in 2007 were all well above the average of 1000 kg/ha seen in most commercial dill fields - this likely reflects the near ideal conditions in 2007, coupled with the extra care taken to reduce seed loss due to shattering.

In the 2006 trial several of the DH lines had yields that were substantially greater than the industry standards (CDC Giant) or the parental line (Mammoth). In 2007, none of the DH lines outperformed the standards. This may reflect the fact that growing conditions through the fall of 2007 were near ideal – this would have been advantageous for the relatively late maturing lines like CDC Giant and Mammoth. In years with an earlier or less favorable fall the earlier maturing DH lines would likely produce better yields and as the seeds would be more mature, the oil content would also be expected to be higher.

The seed was air dried after harvest and then cleaned using a dockage tester. Quality analyses of the essential oils extracted from the seed were conducted as previously described.

Table 2007-1. Agronomic observations on double haploid and industry standard lines of dill in 2007).

Line	Stand	Herbicide Damage ^a	Flowering % (July 18)	Yield (kg/ha)	1000 seed wt. (g)	Essential Oil (%)	Linonene (%)	Oil yield (kg/ha)
DH-7	Good	M/S	0	2750ab	1.89a	3.03b	54.8	83.3ab
DH-12	Good	L/M	5	2851ab	1.70b	2.81c	53.9	80.1b
DH-35	Good	M	0	2962a	1.50c	2.89c	52.2	85.6ab
DH-45	Fair	M/S	0	2357c	1.55c	2.76c	52.5	65.1c
DH-47	Poor	M	0	1412d	1.66b	3.11a	51.8	43.9d
DH-53	Good	M	0	2616b	1.48c	3.12a	53.4	81.6b
Mammoth	Good	M	2	3136 a	1.66b	2.82c	50.5	88.4a
CDC Giant	Good	L	5	3226 a	1.75b	3.05b	48.4	98.4a
<i>Average</i>	-	-		2669	1.65	2.94	52.2	78.3

^a L = low, M=moderate, S=severe.

^e Values within columns followed by the same letter are not significantly different ($p=0.05$)

Notable performance and yields characteristics of the DH dill lines tested in 2007 were;

- a. Line DH-12 produced yields that were within 10% of the industry standard lines, yet it was ready for harvest more than a month earlier (August 23 versus October 6) than the standard lines. DH 12 again produced a vigorous stand of fast growing uniform plants. While it had showed significant herbicide damage in the 2006 trial, there was relatively little damage in 2007. This suggests the importance of developmental stage in determining sensitivity to linuron. DH 12 is far shorter in stature than the standard lines. A shorter plant stature may be desirable in rank crops like dill as it reduces the mass of material to be combined. In the 2006 trial, line DH 12 experienced severe yield loss to what was thought to be blossom blight. In the 2007 trial no blossom blight was observed, yet there was again a fairly high incidence of aborted or shrunken seed in DH 12. Flowering of DH 12 is quite synchronous and occurs when the plants are relatively small. It is possible that the apparent issues with seed set simply reflect the plants' inability to fill the huge number of seeds that set at one time. Selecting for synchronous flowering at an early growth stage may be a yield limiting strategy – however it tends to guarantee at least some yield. Seed of DH 12 was quite large, again suggesting a high degree of maturity.
- b. Line DH-47 is another short stature line that had performed well in early trials. However the seed of DH 47 generated in 2006 had been of poor quality and that resulted in a poor stand and limited yields in the 2007 trial.
- c. CDC Giant – had problems with herbicide toxicity and blight in 2006, but in 2007 it produced the highest yields of the lines tested.

- d. Lines DH-7 and DH-53 – in 2007 these lines again produced vigorous stands of uniformly tall plants. Both of these lines were relatively late maturing and their yields were substantially lower than industry standards which are also late maturing. DH-7 produced the largest seeds of any line tested in 2007.

Seed Quality in 2007 (see Table 2007-1)

Overall the essential oil content of the seed harvested in 2007 was slightly higher than in 2006, reflecting the greater degree of crop maturity achieved in 2007. The seed harvested in 2007 had a higher limonene content than seen in 2006 – the reason for this quality shift is not clear. Within the lines tested in 2007, there was less variability in the oil composition (% limonene) than in the oil content. Oil extracted from CDC Giant appeared to have a lower limonene content than any of the other lines tested in 2007. Oil yields (kg/ha) were far higher in 2007 than in 2006. This reflects the higher seed yields in 2007 rather than any improvement in seed oil content.

While line DH-12 had the highest seed oil content in the 2006 trial, its seed oil content was amongst the lowest of the lines tested in 2007. This emphasizes the importance of multi-year trials when evaluating any germplasm. DH-53, which is quite late maturing, had the highest seed oil content in both the 2006 and 2007 trials.

Caraway in 2007

The 2006 field trials with DH annual caraway had produced very limited seed yields, and the seed was of poor quality. This strongly suggests that the growing season in Saskatchewan is not long enough to consistently mature the presently available lines of annual caraway. It is noteworthy that the 2006 crop failed to mature despite the fact that the crop received a head-start by transplanting coupled with the fact that 2006 was an exceptionally warm year.

The 2007 field trial with DH caraway was conducted adjacent to the DH dill trial, at the previous described University of Saskatchewan research site. All variables and procedures were identical to those described in the dill trial except;

- the trial was seeded using the DH caraway lines that had produced appreciable amounts of seed in 2006. This process strongly selected for fast maturing lines.
- due to limited quantities of available seed, the trial was only planted out in three replicates and each replicate consisted of only two rows rather than the three rows used in the dill trial.
- as annual caraway is extremely late maturing, the crop was left in the field until mid-October. It was then direct combined. The delayed harvest may have resulted in some seed loss to shattering. Delaying the harvest until this point in the season would be extremely risky for a commercial grower

Results

The caraway was slower to emerge than the dill and the stand quality of the various lines was highly variable (Table 2007-2). Stand counts for the parental lines were not appreciably different than for DH 10 and 29. The caraway appeared to suffer fewer ill effects from the linuron spray than the dill. This may reflect the smaller size of the caraway plants at the time of spraying.

Table 2007-2. Field performance of DH annual caraway lines tested in 2007.

Line	Parent	% stand	(%) Aster Yellows	Yield (kg/ha)	1000 seed wt. (g)	Seed oil (%)	Limonene (%)
DH10	NN-2	64	40	1184	3.98	3.06	56.7
DH21	Moran	8	66	41	5.64	2.77	57.5
DH29	NN-2	50	64	488	5.52	3.16	57.4
Parental	NN-2	40	50	270	4.52	3.09	53.3
Parental	Moran	43	62	187	4.85	3.70	56.1

In mid-June it became apparent that there was a severe problem with aster yellows in the entire research plot area – including the caraway plots. We had seen increasing levels of this disease in 2006, but did not suspect that the problem would persist into 2007. Nonetheless, the caraway crop was heavily infected and the effect on the plants was much more severe than in dill or coriander – and much more like the devastating effect seen in carrots. The plants became yellow and produced multitudes of small distorted leaves and stems. None of the infected plants survived to produce seed and as a consequence, seed yields in this trial were compromised. There did not appear to be any significant difference in the incidence or impact of aster yellows for the various DH lines or the parental lines of caraway.

Given the problems with stand establishment, and aster yellows, seed yields were actually unexpectedly high – exceeding by a substantial margin the yields typically seen under commercial production. It is risky to extrapolate small plot yields to field scale operations – but the results are promising. Of particular interest is the fact that line DH 10 out-yielded the line it was derived from (NN-2) **by a factor of 5 fold**. In both the 2006 and 2007 trials, DH 10 had produced a better stand than its parent. In the 2007 trial, this stand advantage was further enhanced by the fact that DH 10 appeared to be less susceptible to aster yellows than NN-2. Of note is the fact that the seed of DH 10 is substantially smaller than the seed of any of the other DH lines or the parental lines. This may be undesirable for sales direct to the consumer where large seed size is equated with quality – however it would be of little importance to the processing sector.

Quality analyses of the 2007 caraway crop were completed as planned. The seed oil content was much higher in 2007 than in 2006 – this likely reflects the fact that the crop was allowed to mature late into the fall of 2007. The seed oil content of the DH lines was comparable to the parental lines – although the variability between lines was greater than that seen in the dill trial. Again the oil composition (% limonene) was more stable across the lines tested than the actual oil content. The highest yielding DH caraway line (DH 10) had a moderate seed oil content – but for total oil produced/ha it would clearly have exceeded all other lines, including its parent.

Greenhouse trials of new DH lines

The process of creating double haploids yields only a limited number of seeds. It is therefore imperative that this seed is planted out and multiplied under near-ideal conditions. For that reason we conducted greenhouse grow out trials over the summer of 2007, to evaluate some of

the new DH lines generated by Dr. Ferrie. The seeds were sown out in late May and their germination was evaluated along with their growth habit.

Table 2007-4. Germination and growth characteristics of new DH lines of various spice crops.

Crop	Parent	Line	% germination
Dill	Hercules	DH 57	31
Dill	Fernleaf	DH 58	7
Anise	Sweitzer 92	DH 21	0
Anise	Sweitzer 92	DH 22	0
Anise	Sweitzer 92	DH 23	0
Anise	Sweitzer 92	DH 24	0
Anise	Sweitzer 92	DH 25	0
Anise	Sweitzer 92	DH 26	0
Anise	Sweitzer 92	DH 27	0
Caraway	NN-1	DH 30	33
Caraway	NN-1	DH 31	10
Caraway	NN-2	DH 32	0
Caraway	Moran	DH 33	0
Caraway	Moran	DH 34	62
Caraway	Moran	DH 35	0
Caraway	Moran	DH 36	53

Results – As has been observed previously, many of the DH lines failed to germinate – this is common in DH of other crops and reflects the fundamental change in genetics of DH plants. Anise appeared to be very prone to lethal effects in the DH – but some viable DH lines of anise have been generated in the past by Ferrie et al.

The plants that did germinate were taken through to maturity and the resulting seed is available for use in future screening trials.

2008 - DH Dill Trials

The 2008 trial focused on the DH lines tested in 2007 as no new lines tested in 2007 had shown sufficient promise to merit inclusion in this trial.

Unless otherwise specified, the procedures utilized in the 2008 DH dill trials corresponded to the procedures previously described for the 2007 trials. The trial was again conducted at the University of Saskatchewan Horticulture Research Facility in Saskatoon. The test site had been in lettuce and onions in 2007 – this resulted in relatively limited weed pressure – except for common groundsel which has become problematic throughout the test plots. The field was prepared for planting as previously described. The trial was seeded in the 3rd week of May. This is a later planting date than most commercial growers would use - but it reflects the fact that the clay soil at the test site is very slow to dry and warm in the spring. The crop was again seeded with a push-type small plot seeder. Each line was planted out in blocks of 4 rows, with each row

being 6 m long. The between row spacing in the 2008 trial was reduced to 0.3 m as compared to 0.5 M in previous years. The tighter row spacing was used to produce a thicker crop canopy earlier in the season, thereby potentially reducing problems with weed competition. Each plot was separated by a 1 m buffer. The trial was laid out in a randomized complete block design with 4 replicates.

The seed used in the 2008 trials was generated in the 2007 trial. Although the germination % of the seed was relatively good, we still opted to seed relatively heavily because of the heavy nature of the soil at the test site. This resulted in a dense stand for most lines except DH 7 and 45. By July, the crop canopy for the various DH dill lines appeared quite uniform – despite the difference in initial plant stand. This reflects dill's indeterminate growth habit.

Cool weather through late May and most of June resulted in extremely slow and uneven crop emergence. Some plants came up within 2 weeks of planting, but the majority took over a month to emerge. This delayed emergence response appeared to be consistent across lines and replicates. Although conditions were not favorable for emergence of the dill crop, they were suitable for the germination of weeds – particularly common groundsel. A healthy weed population coupled with slow crop emergence resulted in heavy weed pressure throughout the plot area. In the 2006 trial the herbicide linuron (Lorox) applied at 200 g a.i./a once the crop reached the 3 true leaf stage had caused significant crop damage. In the 2007 trial using Linuron at a lower rate (150 g a i./a) had reduced crop damage but also provided less effective weed control. Because of the heavy weed pressure in 2008 we opted to return to the higher rate of Linuron. This resulted in little crop damage and decent weed control, except for the common groundsel. The groundsel was so advanced by the time that the dill crop was finally ready to spray that the groundsel went to seed before the herbicide became effective. As these weed plants collapsed and died they released their seeds. These seeds were incorporated into the soil during the supplementary tillage operations required to clean up weed escapes. These weed seeds germinated following each rain event – creating flush after flush for the duration of the growing season. These weeds were controlled by hand tillage until the dill crop grew to the point where it shaded out the small stature groundsel plants.

Because of the problems with blossom blight observed in 2006, we again opted to implement a preventative spray program in 2008. The crop was sprayed with azoxystrobin (Quadris) or clorthalonil (Bravo) every 10 days from mid-July as the crop began to come into flower through until mid-August by which time the crops had begun to mature. There were no obvious signs of blossom blight in 2008 – whether this indicates the spray program was effective could not be determined as there were no untreated areas for comparison. Although there was no disease apparent, some lines again flowered well, yet set relatively few seeds and many of those seeds were shrunken and of low quality. Whether this problem reflects a disease or is a varietal response to adverse growing conditions at flowering could not be determined.

Seed loss due to shattering, both prior to harvest and during combining, is a major issue during the harvest of dill. In 2006 the crop was straight cut combined once it had matured – but this resulted in significant shattering loss, particularly in early maturing lines or in situations where die-down was delayed by cold, wet weather. In 2007 we had tried chemically desiccating the crop, followed by swathing 5 days later and then combining after another week of dry down. Although this harvest procedure did not completely eliminate shattering loss, the losses were far lower than in previous years. In 2008 we tried to further refine this process. We chemically desiccated the crop as before, but allowed it to dry standing and then straight combined the crop. The thought was that the standing crop would dry out more quickly and that elimination of the swathing step might also reduce total loss to shattering. **Unfortunately conditions in the fall**

were less than ideal for this approach to crop management. All lines were slow to mature in 2008 and therefore the desiccation and dry down step occurred during relatively cool weather. This slowed the drying process, leaving the standing crop exposed to shattering loss for extended periods of time. **This problem was most severe on the late maturing lines (DH 7 and 53) which stood for more than 3 weeks before they were dry enough to combine.** Although it was not possible to quantify shattering losses, visual inspection of the field suggested that it was substantial.

Despite problems with weed competition and shattering losses, seed yields in 2008 were all well above the average of 1000 kg/ha seen in most commercial dill fields.

In the 2006 trial several of the DH lines had yields that were substantially greater than the industry standards (CDC Giant) or the parental line (Mammoth). In 2007, none of the DH lines outperformed the standards. In the 2008 trial, the early maturing DH lines (DH 12 and 35) had yields that were significantly higher than the standards. Yields of the later maturing lines (DH 7 and 53) were reduced due to shattering loss.

The seed was air dried after harvest and then cleaned using a dockage tester. Quality analyses of the essential oils extracted from the seed were conducted as previously described.

Table 2008-1. Agronomic observations on double haploid and industry standard lines of dill in 2008).

Line	Stand	Herbicide Damage ^a	Yield (kg/ha)	Essential Oil (%)	Linonene (%)	Oil yield (kg/ha)
DH-7	Good	L	2000 d	2.21 c	65 a	44.2 d
DH-12	Good	L	3110 a	3.10 a	60 b	96.4 a
DH-35	Good	M	3360 a	2.45 bc	58 bcd	82.3 b
DH-45	Fair	M	2700 bc	2.16 c	55 cd	58.3 cd
DH-47	Fair	M	3040 ab	2.64 b	57 bcd	80.2 b
DH-53	Good	L	1890 d	2.50 bc	59 bc	47.2 d
Mammoth	Good	K	2280 cd	2.48 bc	58 bcd	56.5 cd
CDC Giant	Good	L	2870 ab	2.36 bc	55 d	67.8 c
<i>Average</i>	-	-	2656	2.48	58	66.4

^a L = low, M=moderate, S=severe.

^e Values within columns followed by the same letter are not significantly different ($p=0.05$)

Notable performance and yield characteristics of the DH dill lines tested in 2008 were;

- a. DH 35 has emerged as the line that most consistently delivers yields superior to the existing standard lines. It produces a vigorous stand of moderate stature plants that are ready to harvest about 1 week before the standard lines.

- b. Line DH-12 produced yields in 2008 that exceeded the industry standard lines, and it was ready for harvest two weeks earlier (Sept 10 versus October 1) than the standard lines. In previous trials DH 12 had shown superior vigor early in the season. This was also apparent in 2008 – where a portion of the seeds germinated within 2 weeks of planting despite less than ideal field conditions. Although these plants got off to an early start, the majority of the DH seedlings took over a month to emerge. This resulted in a very uneven crop for DH 12 in 2008 – whereas in other years this line had been exceptionally uniform. Seed from the early emerging plants of DH 12 was lost to shattering before the rest of the plants in the DH 12 plots were ready to be desiccated.
- c. Line DH-47 is another short stature line that had performed well in 2008. This line had produced a poor stand in the 2007 trial but produced an acceptable stand in 2008.
- d. As usual, the DH-7 and DH-53 lines produced vigorous stands of uniformly tall plants. Both of these lines were relatively late maturing and their yields were reduced due to shattering loss in 2008.

Seed Quality in 2008 (see Table 2008-1)

Overall the essential oil content of the seed harvested in 2008 was slightly lower than in 2007, but was comparable to 2006 – this reflects the relatively greater degree of crop maturity achieved during the exceptionally long and warm 2007 growing season. The seed harvested in 2008 had a higher limonene content than seen in 2006 or 2007 – the reason for this shift is not clear. Within the lines tested in 2008, there was again less variability in the oil composition (% limonene) than in the oil content. Oil extracted from CDC Giant appeared to have a lower limonene content than any of the other lines tested in both 2007 and 2008. Oil yields (kg/ha) in 2008 were lower than in 2007 but higher than in 2006. Year to year differences in seed yield have a greater impact on oil yields/unit area than differences in seed oil content. .

DH-12 had the highest seed oil content in the 2006 and 2008 trials, but its seed oil content was amongst the lowest of the lines tested in 2007. DH-53, which is quite late maturing, had the highest seed oil content in both the 2006 and 2007 trials, but in the 2008 trial its seed oil content was near the mean. These year to year differences in performance emphasize the importance of multi-year trials when evaluating any germplasm.

Caraway

The 2008 field trial with DH caraway was conducted adjacent to the DH dill trial, at the previous described University of Saskatchewan research site. All variables and procedures were identical to those described in the dill trial except;

- the trial was seeded using the DH caraway lines that had produced appreciable amounts of seed in 2006 and 2007. This process strongly selected for fast maturing lines.
- as annual caraway is extremely late maturing, the 2008 caraway crop was left until mid-September before it was desiccated and harvest was delayed until mid-October. This process may have resulted in some seed loss to shattering. Delaying the harvest until this point in the season would be extremely risky for a commercial grower

Results

The caraway was even slower to emerge than the dill in 2008 and as noted with the dill this led to problems with weed competition. Stand counts for the parental lines were not appreciably different than for DH 10 and 29 (data not shown). As noted in previous years, the caraway appeared to be more tolerant of the linuron spray than dill. This may reflect the smaller size of the caraway plants at the time of spraying.

As with the 2008 dill crop, the linuron treatment of the 2008 caraway plot came too late to provide adequate control of the dominant weed in the plot area – common groundsel. While the dill crop outgrew the relatively short stature groundsel plants by mid-July, the shorter caraway plants had to be hand weed for the duration of the 2008 crop season.

Table 2008-2. Field performance of DH annual caraway lines tested in 2008.

Line	Parent	Yield (kg/ha)	Seed oil (%)	Limonene (%)
DH10	NN-2	1080	3.81	68
DH29	NN-2	670	3.65	68
Parental	NN-2	920	2.86	67
Parental	Moran	740	3.43	66

Aster yellows was less of a problem in the 2008 caraway crop than in 2007, with only about 10 % of the plants showing signs of infection. As the infected plants are too small and chlorotic to produce a significant number of seeds, yields may have been reduced due to this problem.

Given the problems with stand establishment and weed control, seed yields in 2008 were actually unexpectedly high – exceeding by a substantial margin the yields typically seen under commercial production. Line DH 10 again out-yielded the line it was derived from (NN-2), although by a far smaller amount than was observed in 2007.

Quality analyses of the 2008 caraway crop were completed as planned. The seed oil content was higher in 2008, than in either 2006 or 2007 – this likely reflects the fact that the crop was allowed to mature late into the fall of 2008. The seed oil content of the DH lines was higher than the parental line (NN-2). Again the oil composition (% limonene) was more stable across the lines tested than the actual oil content. The highest yielding DH caraway line (DH 10) in the 2008 trial also had the highest seed oil content. Oil yield (kg/ha) for the best DH caraway line (DH10) was 50% higher than for the parental line (NN-2).

Conclusions

In replicated field trials of DH dill conducted from 2006 through 2008 this project identified several lines that differed greatly from the parental lines in important agronomic characteristics such as speed of crop emergence, crop stature and time to maturity. These traits may be of value in a breeding program, especially as they are present in a homozygous form in the DH lines. We

also identified several DH lines that were superior in seed and seed oil yield relative to the parental line and the most widely grown commercial dill variety. These lines may have immediate market value. We were also able to identify one line of DH annual caraway that was again clearly superior to presently grown varieties for seed yields and quality. However this line still required a longer growing season than is available in Saskatchewan – and therefore the market for this improved DH line of annual caraway lies elsewhere.

Opportunities for commercial release of the most promising DH lines identified in this project will be pursued by the Crop Development Center of the University of Saskatchewan working in collaboration with PBI/NRC and the Saskatchewan Herb and Spice Growers Association. The DH dill and caraway lines with traits of potential value in crop improvement programs will be preserved and made more widely available through the Plant Gene Resource Center of Agriculture Canada.

Objective 2. Cumin Improvement

a. Double Haploidy Trials

Microspore embryogenesis represents a critical first step in the development of double haploid (DH) lines of cumin. The basic procedures used to establish microspore cultures of cumin were as follows;

Microspores were obtained from cumin plants grown in controlled environmental chambers. When the plants had reached the flower bud stage, the buds were harvested. Large open buds were discarded along with any leaf material. The closed but mature buds in a cluster were placed in a small stainless steel (Lipshaw) basket and sterilized in 70% ethanol for 1 minute then rinsed in sterile water for 1 minute. The buds were then further sterilized by soaking for 15 minutes in 70% Javex (sodium hypochlorite) with Tween as a wetting agent. The buds were then rinsed in sterile water 3 times for 5 minutes. The buds were placed in a Waring blender cup containing a wash medium (½ strength B5-13% sucrose; Gamborg 1968) and blended for approximately 10 seconds. The mixture was poured through a sterile 41 µm Nitex screen into a sterile 50 mL Falcon tube and centrifuged at 1300 rpm for 3 minutes. The supernatant was poured off and the pellet containing the microspores was resuspended in 5 mL of wash medium and centrifuged. The washing of the pellet was repeated three times to get a clean microspore suspension. Between the second and third wash, the density of the microspores was determined. The microspores were then suspended in a suitable culture media at a density of about 50 000 cells/mL (unless otherwise specified). 5 mL aliquots of this suspension were plated in petri dishes containing various culture media. Unless otherwise specified, the cultures were incubated at 32°C for 3 days and then held at 24°C for the remainder of the experiment.

A number of factors potentially influencing embryogenesis by cumin microspores were evaluated in this project;

Genotype: Some genotypes are better suited than others to serving as microspore donors. Ideally the donor plants should thrive under controlled environment conditions and go on to produce large numbers of viable microspores. Several of the cumin genotypes identified by Dr. Waterer as being suited to field conditions in Saskatchewan were evaluated for their suitability as microspore donors (Indian, Syrian, Cruz, Royston Petrie, and Bolier lines). Cumin proved to be difficult to grow in controlled environments. It is extremely susceptible to insects (aphids, thrips, and spider mites), fungal diseases (*Fusarium* wilt) and lodging. Some lines were more prone to lodging than others. While the Indian line was susceptible to wilt disease and prone to lodging, it also had the greatest microspore yield and consequently it was used as the microspore donor in most subsequent experiments.

Donor plant conditions: Donor plants were grown at four different temperatures (22/18°C, 20/15C, 15/10C, and 10/8C). Plants grown under the 15C day/10°C night regime were the healthiest and gave the best microspore yield. The plants grown under the higher temperature (22/18C) resulted in a microspore preparation that had accumulated a lot of starch. This is usually a sign of a non-embryogenic culture. Based on these results, we subsequently grew all donor plants at 15/10C. Various different growth media were tested in an effort to maximize the health and vigor of the donor plants. Sterilized sandy loam soil, Sunshine Mix #3, RediEarth and mixtures of sandy loam soil and Sunshine Mix #3 all proved suitable. A slow release fertilizer (Osmocote 100) was added to the growth material and the plants were watered with a dilute nutrient solution.

Microspore Staging and Bud Size: The optimal stage of the microspore for embryogenesis can vary depending of the species. Usually, the most responsive stage is the late uninucleate to early binucleate. The ideal bud size for cumin was determined to be medium size (small buds with tips of white or pink petals visible).

Media constituents: The composition of the culture medium plays a large role in influencing embryogenesis by microspore. Several basal media were evaluated for their suitability for culturing cumin microspores: NLN (used in the *Brassica* species; Lichter 1982), AT-3 (used in tobacco tissue culture; Touraev et al. 1996), N6 (used in cereal anther culture; Chu 1978), KM (used for cereal tissue culture; Kao et al. 1975), NN (Nitsch and Nitsch 1969), R92.01 and R92.25 (used for culture of chicory; Theiler-Hedtrich et al. 1996), FHG (used in barley anther culture; Kasha 2001), SH (Schenk and Hildebrandt 1972), and Van Den Bulk (used in culture of tulip microspores; van den Bulk et al. 1994). NLN was observed to be the most responsive basal medium, as many swollen microspores were observed. Some microcalli was also reported when R92.25 was used.

Different carbohydrates and carbohydrate concentrations were also evaluated (ie; sucrose versus maltose at concentrations from 5 – 40%). For some species (e.g. *B. rapa*, *B. juncea*), it is beneficial to induce the microspores in a high carbohydrate media; then for continued development the carbohydrate concentrations in the media are reduced. We initiated our microspore cultures in NLN medium with 25 - 40% sucrose or in AT-3 medium with 28% maltose. After 1, 2, or 3 days, the media was removed and replaced with either NLN with 13% sucrose or AT-3 with 10% maltose. There was very little growth response of the microspores in these experiments. A few swollen microspores and some divided cells were observed but no further development occurred.

Culture conditions: Culture conditions after the microspores are isolated can have an effect on microspore embryogenesis. A heat shock is used in many species to shift the microspore to a sporophytic developmental pathway. In a series of experiments, we evaluated four temperature regimes (24, 32, 35, and 40°C) for five different time periods (0, 3, 7, 10, or 14 days) prior to transferring the culture to 24°C for the remainder of the experiment. Higher temperatures (35 and 40°C) and long periods of time (7, 10, and 14 days) had detrimental effects on the microspores. The most responsive conditions (i.e. swollen cells, a few cell divisions) were observed at 32°C for 3 days, however no further development occurred.

Microspore Density: The density of the microspores in culture can affect microspore embryogenesis. Six different cell densities were examined (100 000, 50 000, 25 000, 20 000, 12 500, and 10 000 cells/mL) for microspore culture response. The highest response, i.e. swollen microspores, was observed in cultures with 50,000 cells/mL.

Osmoticum: Osmotic shock has been shown to enhance microspore development in some plants. An osmotic shock treatment was applied to cumin microspores by first starving the microspores of carbohydrate for three days and then supplying sucrose at a final concentration of 10% or 25% sucrose. Some swollen microspores were observed but no further development occurred in response to this shock treatment. PEG (polyethylene-glycol) was also used in initial culture (NLN 10% PEG) for 3 days, after this time sucrose was added to give a final concentration of 20% sucrose. The microspores in the PEG experiments produced pollen tubes, which is not beneficial. The addition of mannitol (0.3M) to NLN with 5, 13 or 25% sucrose served as an osmoticum also. Mannitol was also added to AT-3 25% sucrose but produced no

response. The mannitol was observed to have no detrimental effects on the microspores and it appeared to reduce the amount of free starch in the culture. The microspores cultured in NLN 25% sucrose with mannitol had the most swelling but no further development was observed.

Cysteine, Brassinosteroids and PCIB: According to Grewal et al (2006), treatment with cysteine at 40 mg/L was found to stimulate androgenesis and plant regeneration. Microspores from the Syrian, Indian, and Richters lines of cumin were cultured in NLN 25% sucrose and 0.3M mannitol augmented with 40 mg/L cysteine. Swollen microspores were reported but no further development occurred. Brassinosteroids are known to increase microspore embryogenesis in the *Brassica* species (Ferrie et al 2005). Brassinolide was added to NLN 25% sucrose at a concentration of 10^{-6} M. The brassinolide treated cultures were incubated at 32°C for 3 days or 24°C constant. There was no growth response to any of the treatments. Another chemical said to increase microspore embryogenesis is PCIB (p-chlorophenoxyisobutyric acid), an anti-auxin (Agarwal et al 2006). PCIB was added to NLN 25% sucrose and 0.3M mannitol at a concentration of 20 μ M. Again, this chemical produced no beneficial response.

Electroporation: Electroporation has been used to induce microspore embryogenesis in other species (e.g. asparagus and chickpea). Microspores of cumin were subjected to 1 or 2 pulses at 250 V/cm for a time constant of 1-3 seconds then cultured in NLN 25% sucrose or AT-3 28% maltose. A heat shock was also applied at 32°C for 3 days or 24°C constant. The microspore viability was tested using a FDA (fluorescein diacetate) stain and observed using a fluorescent microscope. After electroporation, the microspore viability went down to about 50%. A few swollen microspores were observed in the electroporation experiments but no further development was observed.

a. Seed Mutagenesis Trials

EMS (Ethylmethanesulphonate) is a carcinogenic mutagen that produces random mutations in genetic material by nucleotide substitution. The objective of this part of the project was to mutagenize cumin seeds, grow the plants and evaluate for phenotypic differences. Cumin seed (Indian line) were exposed to varying concentrations of EMS (0.5, 1, 1.5, and 2%) for varying time periods (2, 4, and 6 hours). Seed germination rates and phenotypic variations in the resulting plants were observed. A dose-response curve was developed to determine the optimum concentration of the mutagen. Germination of the treated seed decreased as the concentration of EMS and the duration of exposure to the mutagen increased. When the resulting mutagenized seed was grown out no morphological differences in the resulting plants were observed, even at the highest dosages of EMS tolerated by the cumin seed.

Conclusion

This project clearly indicates that cumin is highly recalcitrant when it comes to androgenesis. Although **over 900** different treatment combinations were evaluated in a series of embryogenesis trials (donor genotype, donor plant conditions, developmental stage of the pollen grain, culture media, culture conditions and shock treatments) at most a few cell divisions were observed. Standard mutagenesis techniques also proved of no value as a means of generating useful variation in existing cumin lines.

Project Extension

Results of this project have been or will be made known to the growers and industry stakeholders through;

1. field days involving tours of the research plots
2. presentations at grower meetings ie; Saskatchewan Herb and Spice Growers Annual meetings and newsletters and via presentations at conferences, ie; International Association for Plant Tissue Culture and Biotechnology, August 13-18, 2006, Beijing, China. (Abstract S145, p.44), Plant Canada (2006), and Int. Conference on Haploids in Higher Plants, Feb 12 – 15, 2006, Vienna, Austria (Abstract p. 14), Tradition to Technology – NHP Research Society of Canada conference, May 2007, Plant Canada, July, 2007.
3. publication via Dept of Plant Sciences Vegetable and Special Crops Research website
4. presentation to industry partners interested in commercialization of new lines
5. Peer reviewed publications:
 - a) Ferrie, A.M.R. (2006) Development of double haploidy in the umbelliferae. *Acta Horticulturae* 725:829-835
 - b) Ferrie, A.M.R., Bethune, T., Kernan, Z. 2005. An overview of preliminary studies on the development of doubled haploid protocols for nutraceutical species. *Acta Physiologiae Plantarum* 27:523-529.
 - b. Ferrie, A.M.R. 2007. Doubled haploid production in nutraceutical species: a review. *Euphytica* 158:347-357
 - c. Ferrie, A.M.R. Current status of doubled haploids in medicinal plants. 2009. In: *Advances in Haploid Production in Higher Plants*. Touraev, A., Forster, B.P., and Mohan Jain, S. eds. Springer.pp. 209 – 217. DOI 10.1007/978-1-4020-8854-4.
 - d. Ferrie, A.M.R. and Bethune, T.D. 2007. Microspore embryogenesis in the Apiaceae. In: *Biotechnology and Sustainable agriculture 2006 and Beyond*. Xu, Z., Li, J., Xue, Y., and Yang, W. eds. Springer, Dordrecht p. 465-468.
 - e. Ferrie, A.M.R., Bethune, T.D., and Waterer, D. Microspore embryogenesis and field evaluation of doubled haploid plants in the Apiaceae: Dill (*Anethum graveolens* L.), Caraway (*Carvum carvi* L.), and Fennel (*Foeniculum vulgare* Mill). Submitted to Botany.

The field plots and the project scientists (Ferrie and Waterer) were featured in a CFQC Farm Gate segment in the summer of 2006.

The support provided by the Agricultural Development Fund for this research was acknowledged in all presentations and written materials.

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