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Results of tissue culture and combining ability analysis on *in vitro* doubled haploid maize lines

Main points of the PhD thesis

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1. BACKGROUND AND AIMS

A new chapter in the history of maize breeding began a hundred years ago, when George Harrison Shull published the first report on the favourable yields and great uniformity of hybrids developed by crossing inbred lines (Shull, 1908). Six years later this author introduced the idea of “heterosis”, for which he used the term “hybrid vigour” (Shull, 1914). The production of hybrids produced from inbred lines began to spread worldwide in the 1930s, gradually replacing open-pollinated maize varieties. The hybrid maize developed using this new technique surpassed the land races previously grown chiefly in terms of yield, grain moisture at harvest and uniformity; this latter trait became particularly important with the mechanisation of agriculture.

Since then, inbreeding has become a basic tool of conventional maize breeding, allowing the development of breeding material that can be maintained in stable form for a long period, and that is clearly distinguishable from other lines, while having a high degree of uniformity.

1.1 Basic problem

During most of the past 100 years hybrids had a much longer “useful life” in commercial production than they do today. As the pace of life speeded up, demand changed, raising new challenges for breeders. One such new requirement is cost efficiency, requiring rapid adaptation to changing agro-ecological and agronomic conditions without losing valuable previous experience. Among the new techniques available, the best-known are plant cell and tissue culture, marker-assisted selection (MAS) and the much debated genetic engineering, all of which have been integrated into conventional breeding.

Using the traditional inbreeding technique 6–8 self-pollinated generations are required for the development of new maize lines. Under normal conditions this means 6–8 years, which can be reduced to half by growing a winter generation in the southern hemisphere. Testing then takes up another 2–3 years, which includes pilot seed production for experimental purposes. It would appear at present that this testing period cannot be shortened any more, but since the mid-sixties other means have been available to reduce the period required for the development of homozygous inbred lines.

In the case of maize the use of the field monoploid method (Chase, 1969) and microspore-derived tissue culture makes it possible to produce lines that are almost 100% homozygous within a year instead of the long cycles of inbreeding previously required.

Progeny tests are still required, but first large numbers of lines of haploid origin must be subjected to genome duplication (doubled haploids, DH) and selection for various breeding purposes.

Opinions on these methods have undergone a considerable change over the last thirty years, and the life-span of commercial hybrids has greatly decreased, making it necessary to integrate the new techniques. The application of these methods allows breeders to respond promptly to changes in market demands and may on occasion also be more cost-efficient that conventional inbreeding techniques.

1.2. Aims

In the course of the research, answers were sought to the following questions:

- How efficient is callus induction and plant regeneration in genotypes specifically selected or developed for the anther culture method optimised in the Cell Biology Department of the Agricultural Research Institute of the HAS?
- How efficient is callus induction and plant regeneration when the method is applied for the “model” genotypes used in tissue culture based on immature embryos?
- Can the narrow time period available for anther culture be widened using a greater range of sowing dates, and does this have any positive or negative effect on the results?
- What is the DH line production frequency in terms of the number of anthers cultured and the total number of calli?
- How do hybrids developed from DH lines originating from tissue culture perform in the field?
- What can progeny tests tell us about the combining ability of the parental lines? Do DH lines developed using this method have any improving effect on the hybrids?
- Do lines produced using the *in vitro* technique differ morphologically (or as regards disease resistance) from conventional inbred lines?
- How stable are the yields of hybrid combinations produced from DH parental lines over different locations and years?
- Can hybrids developed from DH parental components equal the average yields of commercial hybrids (standards), while also having acceptable grain moisture at harvest?
- What prospects are there for the application of the anther culture technique in hybrid breeding?

2. MATERIALS AND METHODS

2.1 Genotypes tested

Two genotypes developed from lines of Chinese origin (DH 109 and DH 105), both of which had good regeneration, and an elite line from Martonvásár, which had a poor response to tissue culture (HMv 5405), were used in the anther culture experiments. A systematic series was developed from these lines, involving direct and reciprocal crosses and backcrosses. Three genotypes widely used for somatic tissue culture (A 188, B 73, Oh 43) were also tested for haploid induction ability, together with the double-cross hybrid 4C1 and three Martonvásár sweet corn hybrids. The effect of sowing date was examined on reciprocal hybrid combinations of DH 109 and the elite line.

2.2 Development of doubled haploids

An efficient method of anther culture and DH production has been elaborated in Martonvásár based on Chinese genotypes with good haploid callus induction and satisfactory regeneration ability (Barnabás *et al*., 1999).

2.2.1 Sample collection

Throughout the work, the explants were anthers originating from inflorescences isolated prior to tasselling on field-grown plants. The development stage of the microspores in the anthers was determined in the laboratory using carmine acetic acid staining.

In the sowing date experiment, the F_1 hybrids were sown on the same dates (May 2, 9, 16, 23) in all four years.

2.2.2 Preliminary treatment of explants

Tassels containing immature anthers were removed from the husks, wrapped in aluminium foil and kept at 7°C for 10 days before culturing (Kovács *et al*., 1992). Only tassel branch sections in the correct stage of development were used in the experiments, i.e. cultures were only initiated from anthers containing microspores in the late uninucleate stage, which then continued to develop during cold treatment. The anthers were surface sterilised, first with 70% ethanol for 2 min, then with 20% hypochlorite solution for 20 min, followed by three rinses with sterile distilled water.

2.2.3 Composition of nutrient media

Haploid induction was examined on F nutrient medium (Yu-Pei medium modified by the addition of 500 mg l^{-1} casein hydrolysate, 120 g l^{-1} sucrose, 0.1 mg l^{-1} 2,3,5-TIBA, 5 g l^{-1} active carbon and 2.5 g l^{-1} Gelrite, Genovesi and Collins, 1982). The medium used for plant regeneration was based on N_6 (Chu *et al*., 1975) supplemented with 1 mg l^{-1} kinetin and 0.5 mg l^{-1} alpha-naphthyl-acetic acid. This medium was used to grow smaller plants, after which they were transferred to N_6O_1 medium, which contained less sugar (20 g l^{-1} sucrose) and no hormones, until an adequate level of root and stem differentiation was achieved.

2.2.4 *In vitro* culture

For the first 28 days the cultures were incubated in the dark at 28°C, after which the developing structures were counted and transferred to plant regeneration medium. Petri dishes each containing 20–30 calli were kept in a growth chamber at 26°C with 16 h illumination per day.

When the shoots reached a length of 0.5 cm the calli were transferred to hormone-free N_6O_1 medium in sterilised glass jars. Rooted seedlings measuring 5–7 cm were then planted into peat cubes and after acclimatisation were grown in a growth chamber at day/night temperatures of 18/15°C with 16-h illumination.

No colchicine treatment was applied in this work. Fertile plants were considered to have undergone spontaneous rediploidisation.

2.3 Field analysis of combining ability

Crossing experiments were carried out in Martonvásár in three consecutive years (2005–2007), using 12 DH maize lines and an elite line from Martonvásár (HMv 5405) as parental components. The testers were Lancaster and Iowa Stiff Stalk Synthetic (ISSS) SLC male plants obtained by crossing Martonvásár sister-lines, and a third tester unrelated to the others (NR SLC).

The performance of the hybrids was compared with the standards used in official state trials for each maturity group.

2.3.1 Experimental design and performance trials

The general and specific combining ability of the DH lines, HMv 5405 and the testers were calculated from the results achieved in Martonvásár, while the results of the performance trials were determined from the data collected from three locations

(Martonvásár, Mezőkövesd and Szarvas) in three years (2006–2008). The yields were converted to 15% grain moisture content.

2.3.2 Morphological description

Each of the crosses made between the 12 DH lines, HMv 5405 and the three testers were sown in several plots in the Martonvásár nursery in each of the experimental years, thus allowing observations on morphological traits to be recorded.

2.4. Statistical evaluation

The basic data were subjected to analysis of variance using the "Agrobase 99® for Microsoft Windows®" computer program, and evaluated according to the guidelines given by Sváb (1981).

3. RESULTS

3.1 Results of tissue culture

The *anther response* of F_1 hybrids with DH 109 as a parental component ranged from 34–40%, while that of their DH progeny was 13–32%. The reason for the wide range of the latter was that, depending on the genetic background, a better anther response was observed for DH lines originating from single crosses between the elite line and the Chinese genotype, while that of lines originating from BC_1 hybrids, backcrossed to the elite line, was poorer.

Experiments on the F_1 hybrids and DH progeny of line DH 105 confirmed these results, with anther responses of 25–27% for the hybrids and 10–17% for the DH lines. The frequency thus had a similar tendency, but the values were lower than those recorded for DH 109. In agreement with this, a mean anther response of 13% was noted for DH 109, with a lower value of 8% for DH 105. The best anther response for derivatives of DH 109 was recorded for the lines DH 31 and DH 136 and in the F_1 and reciprocal combinations. Poorer results were obtained for genotypes related to DH 105, where the largest number of calli was isolated from the anthers of the F_1 hybrids.

In these experiments a response to tissue culture was also given by the Martonvásár elite line. A total of 16 calli (1.8%) were induced from the anthers of HMv 5405, suggesting that even elite genotypes may be capable of a low level of callus induction.

With respect to *callus induction* the F_1 combinations generally gave the best results, but the hybrid superiority and reciprocal effect were not always exhibited unambiguously. A morphogenic response was given in 11% of cases for the DH 109 hybrids and in 9–12% for DH 105. While the morphogenic response is indicative of slight differentiation on the surface of the calli, *green plant frequency* is a more specific expression of the regeneration of whole plants (with leaves, stem and roots) in terms of total number of calli. F_1 hybrids of DH 109 origin had a plant regeneration efficiency of 3–3.5%, while this figure was 0.5–1.5% for their DH progeny. The only exception was DH 141, where 7.8% of the calli regenerated into whole plants. Among the genotypes, DH 136 and DH 31 had lower regeneration efficiency than expected. In the case of DH 105 derivatives, the DH lines generally gave better results than the F_1 hybrids. However, even in this case the frequency of green plants was 1.5–3.5%.

Some 60–80% of intact plants of DH 109 origin yielded 10 or more grains, so these were regarded as *fertile*. The only exception was DH 141, where this ratio was 45%. The corresponding ratio from DH 105 and its progeny ranged from 30–60%.

The callus induction ability of the *model genotypes* frequently used successfully for immature embryo culture (A188, B 73, 4C1, Oh 43: Green and Phillips, 1975; Gengenbach, 1982) and that of various sweetcorn genotypes from Martonvásár was also tested using the DH method. The results indicated that the majority of these genotypes had an induction frequency of less than 1%, the only exception being Oh 43 (2.2 %). In genotypes with a poor response the relative callus induction frequency was 100–225%. In the case of Oh 43, for instance, 62 of the 900 anthers responded, representing 6.9%. Due to the poor morphogenic response, no green plant regeneration was observed.

When plants are raised in the field, all the plant samples for anther culture have to be taken before tasselling, so large numbers of explants all require processing in the laboratory at the same time. The question thus arose of whether the callus induction ability would deteriorate if this period was prolonged by using several sowing dates. The direct and reciprocal variants of an F_1 hybrid of DH 109 origin, which exhibited a reliable, efficient response to anther culture in earlier studies, were used in the experiments.

Averaged over 4 years, the results for four sowing dates clearly showed that delayed sowing had an unfavourable effect both on the anther response and on the absolute and relative callus induction ability. Only slight, non-significant differences were observed between the May 9, 16 and 23 sowing dates, but all three led to significantly poorer values of anther response and callus induction compared with the earliest sowing date, May 2.

Repeating these experiments over 4 years also allowed the year effect to be analysed. Averaged over the four sowing dates, the results indicated that the year had a substantial influence on the anther response. Higher values were achieved in 2005, 2007 and 2008, and significantly lower values in 2006. No significant differences in absolute callus induction ability were observed between either the reciprocal hybrids or the years. Averaged over the four sowing dates, the relative callus induction ability was below 200%, primarily due to the lower callus yield per anther from the three later sowing dates.

3.2 Results of field analyses

A crossing programme was set up in the nursery using the 9 DH lines involved in the tissue culture experiments, together with three other DH lines (DH 384, DH 53 and DH 63). Five of the 12 DH lines were found to improve the GCA values. Of these, DH 56 resulted in an average yield surplus of 0.7 t ha^{-1} in the hybrids. The other four lines (DH 136, DH 143, DH 57 and DH 64) also improved the yield, but to a lesser extent. The mean performance of the DH parental lines in the hybrid combinations did not reach the desired level for the other seven DH lines. The GCA value of HMv 5405, used as a control, surpassed that of the DH lines (1.6 t ha^{-1}) and thus had the best combining ability of all the lines tested.

In this mating system, two of the three testers tended to reduce the yield, averaged over the combinations, while NR SLC led to a 0.5 t ha^{-1} increase in the mean yield of the hybrids. The DH lines formed the best combinations with NR SLC, while the Lancaster and ISSS SLC male parents did not perform well in the hybrid combinations.

In addition to having good combining ability, the DH lines must also pass on satisfactory agronomic and morphological traits. The scoring of external appearance also reflects the adaptation of the given line to Hungarian growing conditions. On the basis of morphological traits it can be said that the DH lines generally differed significantly from the control line (HMv 5405). They were shorter, with fewer leaves above the ear and more below, while the ear attachment height was statistically on par. However, these differences, especially as regards the leaf number, have no practical importance. The mean height of the DH lines ranged from 137–219 cm, which is similar to that of lines developed in Martonvásár by inbreeding (Győrffy *et al*., 1965).

The form of the tassel generally demonstrates the degree of relationship with the exotic parent (Fischer *et al*., 1987). The tassels of well-adapted lines bred under Hungarian conditions generally have 4–10 side-branches, while this value may be 4–50 for genotypes closely related to the exotic material and for land races (Kuleshov, 1955). The average number of side-branches was 8.96 for the DH lines, which is significantly higher than the control value.

A field performance trial was set up at a single location in four replications using a total of 41 genotypes: combinations arising from crosses between 12 DH lines, 1 elite inbred line and 3 testers, and two standard hybrids. As expected, the combined graph depicting yield and grain moisture content at harvest showed normal distribution for both traits. The majority of hybrids with DH parental components (25 combinations) had yields lower than the mean of the standards, but 11 hybrids of DH origin achieved the desired yield level and one hybrid surpassed it, though not significantly. Similarly favourable results were obtained with the two control combinations.

The grand mean of the experiment was 8.9 t ha^{-1}, which was significantly lower than the standard mean (10.5 t ha^{-1}). However, the grain yield alone is not sufficient to determine the value of the hybrid. Consideration must also be given to the grain moisture at harvest.

Compared with the standards, only three of the 39 hybrids had significantly lower grain moisture content, and this was associated with low grain yield. A further 10 hybrids had moisture contents of above 21%, again combined with significantly lower yields, so these did not meet the criteria. Although the grain moisture content of 23 combinations did not differ significantly from the mean of the standard hybrids, 12 of these had yields of below 9.4 t ha^{-1}, which was thus significantly lower than the standard mean.

There were thus a total of 8 hybrids of DH origin that satisfied the criteria and equalled the standard mean for both traits, in one case giving a higher yield than the standards. In the present experiments, these 8 hybrids with DH parental components had yields equal to that of state-registered combinations, with similar grain moisture content at harvest.

The yield stability of the hybrids can be estimated from yield fluctuations over years and locations (Szél, 1998). Yield stability was investigated on a smaller group of DH hybrids in a field performance trials with three replications set up at three locations in three consecutive years (2006–2008). The 14 DH lines and 2 standards were sown in Martonvásár, Szarvas and Mezőkövesd, situated in regions with diverse climatic and soil conditions. Most of the hybrids (11 combinations) did not yield as much as the standard mean, but three combinations had yields that were statistically on par with those of the standard hybrids. The mean yield produced by the standards at multiple locations (9.8 t ha^{-1}) was lower than that recorded in the experiment on the full series of hybrids in Martonvásár (10.5 t ha^{-1}), while the grand mean of the yields for DH hybrids was the same in both performance trials (8.9 t ha^{-1}).

Among the hybrids, 10 had higher grain moisture content than the standard mean, while 4 were statistically on par with the standards. However, none of the four hybrids regarded as satisfactory for this trait was able to equal the standards in terms of yield. Among the combinations tested, the only genotype that satisfied the criteria was DH 56 × NR SLC, which slightly (but not significantly) surpassed the yield of the standards, while its grain moisture content at harvest was only slightly higher than the standard.

4. DISCUSSION (CONCLUSIONS AND RECOMMENDATIONS)

4.1 Conclusions drawn from the results of tissue culture

The anther response of the DH derivatives declined as they became more distant genetically from the original Chinese parent. Nevertheless, the trait is reliably inherited and exhibits a heterosis effect in the hybrids (Wenzel and Uhrig, 1981), being easily transmitted via crossing (Obert *etal*., 1998). In some cases a maternal effect could also be detected in the reciprocal combinations (Tomes and Smith, 1985).

With respect to absolute and relative callus induction, the heterosis and reciprocal effects were not always pronounced and the differences between lines/hybrids were also diverse. The number of calli per anther was genotype-dependent, but was not always correlated with the anther response (Barnabás *et al*., 1988). In general it can be said that if the anther response and relative callus induction ability of a genotype are satisfactory, plant regeneration will probably be successful.

Among the lines tested, the responses of DH 136, DH 31 and DH 141 were favourable. None of the calli induced in the elite line HMv 5405 became differentiated. It is impossible to determine from the experiments whether the low rate of callus induction observed for this elite line is likely to be repeated in other genotypes important in farm practice. It suggests, however, that it is worth searching for other responding genotypes over a wider genetic basis.

Based on the above results, DH 109, which has a 100% Chinese background, had better anther response and callus induction ability than DH 105, so this genotype is recommended for future work on anther culture.

F_1 hybrids having DH 109 as a parental component can also be used to optimise anther culture conditions, as they respond sensitively to changes in culture conditions, while having great efficiency under optimum growth conditions.

When testing the genotypes used for immature embryo culture, it was found that none of them could be used for anther culture with the nutrient media and optimised culture conditions used in the present work, confirming the findings of Brettel *et al*. (1981) and Genovesi and Collins (1982), obtained for other genotypes and culture conditions.

Sowing date had a substantial influence on the anther response and callus induction ability. This can be attributed to the fact that late sowing causes the vegetation period to shift to the beginning of July, when plants are frequently subjected to heat stress and atmospheric drought. This additional stress has an unfavourable effect on the viability of the microspores. These findings are in accordance with those published by Nitsch *et al*. (1982) and Dieu and

Beckert (1986). Genovesi (1990) considered that daily temperature fluctuations could be responsible for the differences observed after sowing at various dates, which influenced the quality of the anthers used for culture.

4.2 Conclusions drawn from field experiments

A total of 12 DH lines and one elite line were crossed with Martonvásár SLC testers with different genetic backgrounds, and the resulting hybrids were included in performance trials in order to calculate values of general and specific combining ability (GCA, SCA), which help to determine the breeding value of the genotypes.

The best combination in the experiment was the HMv 5405 × NR SLC hybrid, where both parents (GCA: 1.6 and 0.5 t ha^{-1}, respectively) improved the value of the hybrid (additive genetic effect; Sprague and Tatum, 1942), while the combination itself also led to a yield surplus (SCA: 0.4 t ha^{-1}; dominant and epistatic effects; Sprague and Tatum, 1942).

Among the progeny of the DH lines, the combinations DH 56 × NR SLC and DH 143 × NR SLC improved the values of both GCA and SCA.

When GCA and SCA values were calculated from the grain moisture content at harvest, 6 DH lines and the elite line HMv 5405 fulfilled the criteria. These again included the lines DH 56 and DH 64, which caused improvements of 1% and 0.5%, respectively, while the tester ISSS SLC resulted in an improvement of 0.5% in the value of the hybrids. The SCA only improved the grain moisture content of two hybrids, over and above the favourable effect of the parents. The other lines that improved the grain yield values, DH 136, DH 143 and DH 57, caused a deterioration in the grain moisture content at harvest. Among the parental lines, DH 56 and DH 64 were thus judged to be satisfactory for both traits.

With regard to morphological traits, the DH lines differed to some extent in appearance, but did not exhibit any fundamental difference compared with inbred lines developed using the conventional technique. In general the initial development of the DH lines was poorer than that of the elite and SLC lines. As expected, due to the slight heterosis effect, the testers were better than the control for this trait.

The performance of hybrids with DH parental components can best be illustrated in terms of yield and grain moisture at harvest. To give a more complete picture, the GCA and SCA analysis of the lines was thus complemented by an evaluation of progeny performance.

Averaged over 14 hybrids studied at several locations in a number of years, it could be seen that the combinations exhibited differences in both grain moisture content and grain yield in response to year and environment effects. The statistical analysis confirmed that the year had a substantial influence on both traits. The grain yield was lower in 2007, an unfavourable year for maize production, and the grain moisture content at harvest was around 19.3%. In the other two years, higher yields were recorded, but the grain moisture content was also higher.

In evaluating the results it must not be forgotten that the experiments were carried out on material with a very narrow genetic background, as the 12 DH lines were derived from crossing combinations between a single inbred line of Iodent origin and an exotic inbred line. It seems likely, therefore, that if the analysis were extended to include DH lines developed on a broader genetic basis and a larger number of test crosses, it would prove possible to select maize hybrids surpassing the standard mean, which could thus be granted state registration.

The location had a considerable influence on the results obtained for hybrids of DH origin. In Mezőkövesd low yields were recorded, while in Szarvas they were much higher, with greater grain moisture content. However, the year had an even greater effect on the yield than the location. The greatest yield fluctuation caused by the year was almost 4 t ha^{-1}, compared with 3 t ha^{-1} for the location. It can thus be concluded from the results that both the year and the location resulted in substantial fluctuations in the yields of the hybrids. This was true not only of the DH lines, but also of the standards. The joint effect of location and year caused variations of as much as 4 t ha^{-1} in the yield and of 4–5% in grain moisture content at harvest.

NOVEL SCIENTIFIC RESULTS

In work on maize anther culture and the evaluation of the regenerated (homozygous) DH lines, the haploid callus induction and plant regeneration ability of the genotypes was determined, and the method was then extended to lines and hybrids used in other tissue culture systems. The effect of delayed sowing on induction ability was investigated, and differences caused by the year effect were recorded.

Field experiments were set up to study the performance of hybrids with DH parental components and the effect of year and location on the yield stability of these hybrids. The results were compared with those achieved for the standards used for each maturity group in official state trials. The data from progeny trials were used to calculate the general and specific combining ability of the lines, which were then compared with morphological data. This combination of field and laboratory data allowed a more comprehensive evaluation of DH lines developed using *in vitro* methods.

In the course of the experiments the following novel results were achieved:

1. This was the first time that a combination of laboratory and field systems had been applied for the evaluation of DH maize lines developed from anther culture.
2. Based on the laboratory and field evaluation of the DH lines it was possible to identify genotypes that not only had a satisfactory level of haploid callus induction ability, but also possessed agronomic traits useful for breeding and led to an acceptable yield surplus in the progeny.
3. The need to isolate anthers from field-grown plants all flowering at around the same time in early July causes an unacceptably high work load, so studies were made to determine whether this problem could be overcome by sowing at different dates. However, delayed sowing was found to reduce the haploid callus induction frequency, thus making tissue culture less successful.
4. The year was found to have a substantial influence on the anther response and, to a lesser extent, on the absolute and relative callus induction ability.
5. The results indicated that the model genotypes (lines, hybrids) successfully applied in immature embryo culture were not suitable for anther culture. This suggests that suitability for tissue culture is not necessarily a general characteristic of the plant, applying to all the plant organs. However, while this is true for the technique used in the

present work, it does not mean that better tissue culture results could not be achieved if the *in vitro* DH technique were further optimised for a given genotype.

6. Performance trials on hybrids developed in test crosses with DH lines revealed that it is possible to develop combinations with values of yield and grain moisture at harvest that are on par with the mean values of standard hybrids. Although the majority of the combinations tested in the experiments did not reach the desired level, this can probably be attributed to the fact that the DH lines had a very narrow genetic background (basically being combinations of two homozygous lines in various proportions). There is thus every likelihood that competitive hybrids could be developed if basic populations with greater genetic variability were employed.
7. The general combining ability values calculated from progeny tests on the DH lines confirmed that the analysis of even a small number of lines may reveal genotypes with an improving effect among the plant materials developed using the *in vitro* technique. This improving effect may be associated with favourable agronomic traits. The lines were also found to be similar in appearance to inbred lines developed in the conventional manner.
8. On the basis of the results, F_1 hybrids of the line DH 109 and their DH progeny (DH 31, DH 136, DH 141) can be recommended for anther culture. In field combining ability analysis, the lines DH 56 and DH 64 improved both the yield and the moisture content at harvest of their hybrids. These two lines also have satisfactory, though not outstanding, haploid callus induction and plant regeneration ability.

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