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# Breeding qualities of monogerm dihaploid sugar beet lines

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## Abstract

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The successful induction of sugar beet haploids through *in vitro* cultivation of unpollinated ovules, followed by chromosome doubling of the regenerants, enables the discovery and stabilization of valuable gene recombinants. The objects of our study are doubled regenerants, obtained by in vitro unpollinated ovules of individually selected hybrid monogerm plants. The pollen fertility of the monogerm dihaploids is high; they exhibit full gametophytic self-compatibility, which allows for their use as "O" types of monogerm MS lines. The higher indices of productivity and technological qualities of the dihaploid lines, respectively, their MS analogues, are a precondition for their better appearance as parental components in hybridization. The combining ability of the tested monogerm dihaploid lines regarding the root yield in their triploid crosses is exceptionally high. The 15-4 line has proven higher productivity in its triploid crosses, and the diploid hybrids of 55-4 yield high amounts of root and white sugar per unit area.

*Keywords:* sugar beet; monogerm; dihaploids; hybrids; white sugar yield

## Introduction

Self-pollinated (inbred) lines are used in sugar beet heterosis breeding. The obtaining of homozygous lines by compulsory self-pollination is associated with some difficulties. The self-sterile biotypes, being a fundamental part of the population, rarely produce seeds. Additionally, bringing genes, part of which are recessive, in a homozygous condition is accompanied by inbreeding depression in the lines. And not least, the inbreeding results in a significant narrowing of the hereditary basis of the initial population (Zakhariev and Kikindonov, 1997). Haploids are plants with a gametic number of chromosomes (n). After doubling the chromosome number, spontaneously or with the use of antimitotic agents, double haploids (dihaploids) are obtained.

The application of the dihaploidy (Bossoutrot and Hosemans, 1985; Van Geyt et al., 1987; Zhuzhalova et al., 2020) in the breeding program significantly shortens the process of obtaining homozygous lines and can replace the long-lasting inbreeding (Atanassov, 1988). The obtainment of plants from gametes, rather than from zygotes, through induced haploidy (Steen, 1987) is an excellent method to identify and stabilize valuable gene recombinations into lines, thereby determining the expression of useful productivity and technological parameters in sugar beet (Nichols et al., 1992). With only one reproductive cycle, covering cultivation in vitro, rooting, adaptation, diploidization of the haploid plants (Ragot and Steen, 1992; Hansen et al., 1994), and seed reproduction, the harmful recessive alleles are eliminated. While with the advance of the inzucht (repeated selfing), the possibilities of differentiation of valuable recombinants are decreased, by induced dihaploidy, these possibilities are practically unlimited in the gametes of $F_1$ hybrids, so the induced haploidy is a highly effective method for enrichment of the gene fund (Kolesnikova et al., 2021).

Among the many applications of *in vitro* culture techniques, sugar beet has benefited the most from haploid plant production, protoplast culture, and somaclonal variation and *in vitro* cell selection (Mezei et al., 2002; Gurel et al., 2008; Seeja and Sreekumars, 2020). Genetic transformation technologies have been developed, and genetically modified herbicide-tolerant sugar beets and rhizomania-resistant transgenic varieties are being evaluated and successfully applied in practice.

With all the advances in molecular biology, traditional plant breeding methods are still needed in the development of sugar beet varieties.

There are many reports with different protocols of induced dihaploidy in sugar beet (Gurel et al., 2021; Lux et al., 1990; Svirshchevskaya and Dolezel, 2000; Zhuzhalova et al., 2020; Vasilchenko et al., 2021), but almost no reports on the use of the obtained dihaploid forms in practical breeding.

Propagation of CMS plants requires a pollen-fertile maintainer genotype (referred to as O-type in sugar beet). Sugar beet plants with the maintainer genotype are comparatively rare (less than 5%) in most beet populations (Bosemark, 2006; Dirim, 2023). Furthermore, the induced dihaploidy is an excellent method for enriching the O-type gene fund, as well as the collection of male-sterile monogerm lines.

Our long-term research aims to study some breeding parameters of monogerm dihaploid sugar beet lines. The inclusion of these lines in the cross-breeding schemes provides insights into their genetic and breeding values.

## Materials and Methods

The initial plants are monogerm hybrids with proven high productivity. The sterilization of the flower branches, the isolation of the seed buds, their cultivation in vitro, and the adaptation of the obtained plants were carried out according to the methodology of Slavova (1993). The diploidization of haploid plants followed the method described by Zakhariev (1973). Pollen viability was determined by the iodine method (Pausheva, 1988). Ploidy analyses were performed microscopically and flow cytometrically, based on the quantitative determination of DNA in a sample of thousands of single cells, expressed in histograms. The crosses of male sterile line with the dihaploid sterility maintainers were made in an isolated band of sunflower, in a 2:1 proportion between the MS line plants and the O-type plants, with removal/scrapping of all the fertile plants in the MS line. The individual crosses are conducted in groups of 2 MS plants: 2 O-type plants, scrapping all the fertile and half-sterile plants among the MS-line, and harvesting separately the desired MS and O-type plants.  The field tests for productivity and technological qualities were conducted in 4-6 repetitions, using block methods, with a harvest plot of 10.8 $m^2$ and a group standard of varieties. The technological analyses are based on the following indicators: dry matter, determined refractometrically; sugar content, determined by the cold digestion method; soluble ash content, determined conductometrically; alpha-aminonitrogen, determined colorimetrically; and K and Na content, determined by a flame photometer. Data from field trials and laboratory analyses were processed statistically (Abramova, 1985; Mather, 1996; Lidanski, 1988).

## Results and Discussion

Our study covers a long period of tests. The first observations were on the fertility of pollen and the monogermity of the regenerants, obtained through *in vitro* cultivation of unpollinated ovules of monogerm hybrids (Table 1). The monogermity of the obtained dihaploids is relatively higher than the monogermity of the Fo generation of the in vitro propagated initial line (81%), even if in the $F_2$ generations, that difference decreases. The dihaploids are highly homozygous regarding the genes responsible for monogermity. The high pollen fertility shown by the $F_o$ and $F_2$ generations of the dihaploids, and of the initial line, is a precondition for their use as maintainers of male sterility in breeding. The collection of our O-types is quite limited, and the induced haploidy provides us with the opportunity to enrich it. The field trial performance of the obtained monogerm dihaploids (Table 2) regarding their productivity and technological qualities proves their breeding value.  The root yield from the tested dihaploids is 103.1% to 105.7% of the initial line's yield of 4504 kgs/da, and all the tested variants are at the standard's level of productivity. The sugar content of the monogerm dihaploid DH13-4 is higher than that of DH15-4 and DH54-4, and is at the Standard's sugar content level. The content of soluble ashes (a negative feature) of the DH54-4 is the highest one, with proven differences towards the remaining dihaploids and initial line, and to the Standard's soluble ashes content. The white sugar yield, as a performance indicator, is the highest for DH13-4, and this is the only dihaploid line with a yield of white sugar comparable to that of the Standard varieties. In summary, there are significant differences between the newly obtained monogerm dihaploids, which are indicative of differences in their genomic background.

Backcrossing is a well-established breeding scheme in which a characteristic is introgressed from a donor parent into the genome of a recurrent parent (Antonov, 1982, 1997). In this process, the functional characteristics/alleles of the DH (dihaploid) maintainers are introgressed into the genome of an MS line. Whether we call them backcrosses or test-crosses with a male-sterile line, the purpose of these crosses is to receive a monogerm male-sterile analogue of the O-type, which would be used further on in the heterosis breeding of sugar beet varieties. Moreover, Table 4 presents some results of such test crosses (which we refer to as satu-

**Table 1. Performance of monogerm dihaploids (in % of the initial line and of the Group standard's values)**

| Variant | Root yield, kg/da | | Sugar content, % | | Soluble ashes content, % | | White sugar yield, kg/da | |
|---|---|---|---|---|---|---|---|---|
| | % of Initial | % of Standard | % of Initial | % of Standard | % of Initial | % of Standard | % of Initial | % of S tandard |
| 13-4 | 104.2 | 93.4 | 104.8 | 96.5 | 96.9 | 100.0 | 110.7 + | 87.3 |
| 15-4 | 104.6 | 93.8 | 96.2 | 88.6 | 99.2 | 102.4 | 98.9 | 78.0 |
| 54-4 | 105.7 | 94.8 | 97.9 | 90.1 | 107.6 | 111.1 | 101.9 | 80.4 |
| 55-4 | 103.1 | 92.4 | 101.5 | 93.5 | 102.6 | 105.9 | 105.2 | 83.0 |
| 76-4 initial | 4504 | 89.6 | 15.0 | 92.1 | 0.543 | 103.2 | 531 | 78.9 |
| Standard | | 5024 | | 16.29 | | 0.526 | | 673 |
| GD 5% | 6.9 | 14.1 | 3.7 | 8.5 | 5.1 | 9.9 | 6.1 | 13.4 |

**Table 2. Sterility of the MS- analogues of monogerm dihaploid lines**

| MS analogue | MS plants, % | Half-sterile plants, % | Fertile plants, % |
|---|---|---|---|
| 13-4 / I cross/ | 68.3 | 31.7 | – |
| /II cross/ | 88.9 | 10.9 | 0.2 |
| Individual cross | 94.2 | 5.8 | – |
| 15-4/ I cross/ | 56.3 | 43.4 | 0.3 |
| /II cross/ | 81.7 | 18.3 | – |
| Individual cross | 97.6 | 2.4 | – |
| 54-4/I cross/ | 71.0 | 28.2 | 0.8 |
| /II cross/ | 87.2 | 12.2 | 0.6 |
| Individual cross | 96.8 | 3.2 | – |
| 55-4/I cross/ | 72.0 | 26.0 | 2.0 |
| /II cross/ | 88.8 | 10.8 | 0.4 |
| Individual cross | 93.1 | 6.9 | – |

rating crosses) for the propagation of male-sterile lines. The assessment of the MS progenies/families of the first crosses of the male-sterile line with the monogerm dihaploids shows 56.3% (DH15-4) to 72.0% (DH55-4) sterile plants in the MS progeny. Half-sterile plants are 26.0% to 43.4%, and an insignificant percentage of fertile plants. The progenies of the next crossing (male-sterile line x dihaploid O-type) have much higher male sterility (from 81.7% for the male sterile analogue of 15-4 to 88.9% for the male-sterile analogue of DH13-4). Therefore, obtaining good MS analogues of the monogerm dihaploids is straightforward and does not require much time. With the so-called individual crosses, the male sterility of the analogues could exceed 93% shortly.

Having the male-sterile analogues of our monogerm dihaploids, we had to test their combining ability for heterosis breeding in sugar beet hybrids. Of course, the diploid hybrids are the priority of European breeding now, and this is based on the better diploid hybrids' resistance to diseases and hybrid seed germination, combined with good technological qualities.

**Table 3. Monogermity and pollen fertility ($C_o$ и $F_2$ generations of monogerm dihaploids)**

| Variant | Number of flower buttons | Multi-germ, % | Dioe-cious, % | Mono-germ, % | Fertile pollen, % |
|---|---|---|---|---|---|
| Initial $C_o$ | 834 | 0.1 | 18.7 | 81.2 | 96.6 |
| 13-4 $C_o$ | 890 | – | – | 100.0 | 93.3 |
| 15-4 $C_o$ | 1120 | – | 0.3 | 99.7 | 95.4 |
| 54-4 $C_o$ | 1360 | – | 0.2 | 99.8 | 97.7 |
| 55-4 $C_o$ | 1380 | – | – | 100.0 | 95.2 |
| Initial $F_2$ | 200 | – | 5.0 | 95.0 | 94.7 |
| 13-4 $F_2$ | 200 | – | – | 100.0 | 95.3 |
| 15-4 $F_2$ | 200 | – | 1.0 | 99.0 | 98.2 |
| 54-4 $F_2$ | 200 | – | 0.5 | 99.5 | 97.9 |
| 55-4 $F_2$ | 200 | – | 0.5 | 99.5 | 94.8 |

As multigerm pollinators in these crosses, we have utilized our multigerm dihaploids, each with desirable characteristics (Table 4). The highest root yield is obtained from the crosses of the monogerm line 55-4, which has proven to have higher values in its hybrid with DH58 (111.7% towards the Group Standard value). The same monogerm dihaploid line has the highest sugar content and randeman, and its diploid hybrids have the highest white sugar yield per unit area (mean of 105.9% of the standard's white sugar yield). We would mark the hybrids of the monogerm dihaploid 55-4 with the multigerm DH58 as the best hybrid combination amongst the tested diploid hybrids.

Our diploid pollinators list is not rich in differing genotypes, and hopefully, the crosses with tetraploid multigerm pollinators will give us a better idea of the breeding value of the obtained monogerm dihaploid lines. Here, the multigerm pollinators (4x) differ significantly in their characteristics, as reflected in the values of their hybrids.

All the tested triploid hybrids (Table 5) have significantly higher productivity (root yield) than the Standard varieties. The triploid hybrids of the DH55-4 are exceeding the Standard values with proven differences (in their hybrid combinations with the pollinators 24 and 985). At the same time, the highest productivity is achieved by the DH15-4

**Table 4. Combining ability of monogerm dihaploid lines in diploid crosses (in % of the Standard)**

| Line | Diploid pollinators | | | | | |
|---|---|---|---|---|---|---|
| | Dh 52 | Dh 57 | Dh 58 | Dh 61 | Dh 63 | Mean |
| | Root yield kg/да 4316 кг/da GD 5%- 8.9 | | | | | |
| 13-4 | 93.6 | 99.6 | 100.8 | 99.5 | 102.6 | 99.2 |
| 15-4 | 98.6 | 100.0 | 104.3 | 103.6 | 103.7 | 102.0 |
| 54-4 | 98.4 | 99.0 | 100.3 | 106.4 | 100.0 | 100.8 |
| 55-4 | 103.9 | 101.3 | 111.7+ | 104.9 | 108.5 | 106.1 |
| | Sugar content % 16.13 GD 5% – 5.6 | | | | | |
| 13-4 | 97.1 | 102.2 | 99.8 | 97.9 | 99.0 | 99.2 |
| 15-4 | 98.5 | 98.7 | 99.4 | 100.0 | 101.2 | 99.5 |
| 54-4 | 98.3 | 95.9 | 97.9 | 100.2 | 103.1 | 99.1 |
| 55-4 | 100.1 | 98.0 | 100.4 | 101.8 | 101.5 | 100.3 |
| | Randeman % 12.81 GD 5% – 7.4 | | | | | |
| 13-4 | 97.0 | 102.8 | 99.9 | 96.7 | 99.3 | 99.1 |
| 15-4 | 97.4 | 98.0 | 99.5 | 99.6 | 101.6 | 99.2 |
| 54-4 | 97.2 | 93.8 | 98.2 | 100.2 | 103.9 | 98.7 |
| 55-4 | 98.2 | 97.0 | 100.3 | 101.9 | 101.7 | 99.8 |
| | White sugar yield kg/da 526 GD 5% – 10.7 | | | | | |
| 13-4 | 90.6 | 102.5 | 100.7 | 96.0 | 101.5 | 98.3 |
| 15-4 | 96.5 | 97.8 | 103.8 | 103.1 | 105.2 | 101.3 |
| 54-4 | 97.2 | 92.9 | 98.4 | 106.4 | 103.7 | 99.7 |
| 55.4 | 101.8 | 98.1 | 112.2+ | 107.0 | 110.5 | 105.9 |

**Table 5. Combining ability of monogerm dihaploid lines in triploid crosses (in % of the Standard)**

| Line | Tetraploid pollinators | | | | | |
|---|---|---|---|---|---|---|
| | 24 | 985 | 46R | 46 Rc | 43 $a_3$ | Mean |
| | Yield of roots 5427 kg/da GD 5% – 10.8 | | | | | |
| 13-4 | 100.6 | 114.3+ | 106.7 | 103.7 | 109.8 | 107.0 |
| 15-4 | 109.6 | 107.3 | 108.0 | 120.6++ | 111.1+ | 111.4+ |
| 54-4 | 104.3 | 108.2 | 105.1 | 102.0 | 103.5 | 104.6 |
| 55-4 | 111.4+ | 118.1+ | 108.0 | 103.3 | 96.8 | 106.3 |
| | Sugar content % 16.38 GD 5% – 4.0 | | | | | |
| 13-4 | 96.8 | 97.9 | 96.2 | 102.5 | 96.6 | 98.0 |
| 15-4 | 101.9 | 99.0 | 97.8 | 95.4- | 97.6 | 98.3 |
| 54-4 | 97.6 | 98.3 | 96.5 | 99.9 | 97.8 | 98.0 |
| 55-4 | 99.1 | 96.8 | 97.8 | 96.0 | 99.9 | 97.9 |
| | Randeman% 12.89 GD 5%- 5.7 | | | | | |
| 13-4 | 95.6 | 97.8 | 94.0- | 103.4 | 96.9 | 97.5 |
| 15-4 | 103.5 | 100.0 | 97.1 | 95.2 | 98.3 | 98.8 |
| 54-4 | 97.4 | 97.7 | 94.6 | 99.6 | 97.7 | 97.4 |
| 55-4 | 99.5 | 95.2 | 97.1 | 93.5 | 100.1 | 97.1 |
| | White sugar yield kg/da 695 GD 5% – 12.4 | | | | | |
| 13-4 | 96.3 | 111.9 | 100.4 | 107.2 | 106.4 | 104.4 |
| 15-4 | 113.9+ | 107.3 | 104.9 | 114.9+ | 109.3 | 110.1 |
| 54-4 | 101.7 | 105.6 | 99.5 | 101.6 | 101.1 | 101.9 |
| 55-4 | 110.9 | 112.6+ | 104.9 | 96.7 | 96.9 | 104.4 |

triploid hybrids – even the average value of their productivity exceeds the Standard's value (111.4%).

The sugar content is significant for breeders worldwide. Moreover, we can say that the results of all tested monogerm dihaploids are excellent for their future use in heterosis breeding. The highest sugar content is registered for the triploid hybrids of DH15-4 (mean of 98.3% of the standard sugar content). This dihaploid exceeds DH13-4 and DH54-4 in its triploid hybrids with the pollinator 24, but has a proven lower sugar content in its hybrid with the 46Rc pollinator (as it does with DH55-4's hybrid with the same pollinator). Furthermore, we see almost the same situation with the randeman of the tested hybrids (proven higher randeman of the triploid hybrids of DH15-4 compared to that of the hybrids of DH 13-4 and DH 54-4), respectively proved higher values of DH13-4's randeman in comparison with the randeman of the DH15-4 and DH55-4 hybrids with the 46Rc pollinator. We observe a significant difference between the tested monogerm dihaploids and their triploid hybrids with multigerm pollinators.

The white sugar yield from the hybrids of DH15-4 is the highest one (mean of 110.1% of the Standard's white sugar yield). Furthermore, this, together with the highest mean values of root yield and sugar content in these tests, shows an excellent combining ability of that monogerm dihaploid in crosses with tetraploid multigerm pollinators. We can also note the high combining ability of DH55-4, as expressed in its hybrids with pollinators 24 and 985 – the yield of roots and white sugar yields of its hybrid combinations significantly exceed the Standard's values.

## Conclusions

The successful induction of sugar beet haploids by *in vitro* cultivation of unpollinated ovules allows the discovery and stabilization of valuable gene recombinants. The high pollen fertility of the monogerm dihaploids, exhibiting full gametophytic self-compatibility, is a prerequisite for their use as "O" types of monogerm MS lines.

The higher productivity and technological qualities of the dihaploid MS analogues contribute to their superior appearance as parental components in hybridization.

The DH15-4 line has demonstrated higher productivity in its triploid crosses, and the diploid hybrids of DH55-4 have yielded high amounts of root and white sugar per unit area.

Both dihaploid monogerm lines can be used to create promising sugar beet hybrids with excellent productivity and quality parameters.

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