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# On the Genetic Improvement of Androgenetic Haploid Formation in *Hordeum vulgare* L.

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**Summary.** Anthers of 55 different spring barley (*Hordeum vulgare*) hybrids and four varieties were cultured in vitro. Microspores of each hybrid gave rise to calluses and subsequently plantlets, from all hybrids, except one. As criteria of microspore responsiveness, callus formation and plant regeneration frequencies were studied in detail. Large differences with regard to these criteria were found, which were traced back to the genotype of the anther donor plant. Callus formation varied between 3.3 and 73.2 per 1,000 anthers plated, whereas green plant regeneration ranged from 0 to 12.7 per 1,000 anthers cultured.

Comparisons of microspore regeneration frequencies of hybrids and their parents indicated that culture responsiveness is a heritable, complex character involving at least two different and separately inherited mechanisms:

1) the ability of microspores within anthers to divide and give rise to calluses and subsequently,

2) the ability of calluses for morphogenesis, to yield green or albino plants.

Because it is heritable, anther culture responsiveness can be transferred to breeding material which is initially non-responsive. This genetic way of improving success in androgenetic haploid production appears to be more realistic than the search for optimum culture conditions.

**Key words:** *Hordeum vulgare* – Haploids – Androgenetic responsiveness – Genetics

## Introduction

The usefulness of haploids for the production of homozygous lines and for more efficient selection within smaller populations is accepted today. However, the techniques for haploid production are – especially in crop plants – not very productive and therefore too risky for a practical application in comparison with conventional breeding. The few examples of plants, e.g. tobacco, potato and barley, where techniques for induction of haploids are relatively successful illustrate the significance of haploid techniques for crop improvement (Nakamura et al. 1973; Deaton et al. 1982; Mendiburu et al. 1974; Kasha and Reinbergs 1980). Apart from tobacco, haploids are mainly produced by interspecific hybridization and chromosome elimination, as this is at present more successful than androgenetic techniques. On the other hand, androgenesis could make use of a broader genetic spectrum per plant when the vast numbers of microspores are compared with egg cells. This advantage is maintained over methods based on haploidy inducing genes, e.g. the *ig* gene of *Zea mays* (Kermicle 1969) or the *hap* gene in barley (Hagberg and Hagberg 1980). For that reason we have concentrated on the androgenetic approach although the *Hordeum bulbosum* chromosome elimination technique has already proved satisfactory in *Hordeum vulgare*.

The practical application of androgenetic haploids is still limited by the small number of haploid individuals recovered from anthers. Therefore, experiments have been conducted to improve success in microspore regeneration. Principally, two approaches may be possible: either a study of physiological factors, such as preculture and in vitro culture conditions (Dunwell 1982), or an investigation of the genotypic factors involved, which we report here.

Firstly, we measured the androgenetic response under identical environmental conditions, and further experiments were aimed to clarify the genetic basis of varietal differences found in the former studies.

## Materials and Methods

As anther donor material, different barley cultivars, breeder's strains and hybrids of variable origin were used. In experi-

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ment 1, 53 spring barley hybrids were taken as anther donors; $F_1$ seed was provided by the Josef Breun Seed Company, Herzogenaurach (West Germany). The parentage of some of the crosses is given in Table 1; uniform cultivars or breeder's strains had been used as parents. From each of the hybrids, three to 12 $F_1$ plants were used as anther donors. As many spikes as possible were taken from each plant for collecting anthers, because in earlier studies with barley cv. 'Dissa' no differenes had been found between the first and subsequent spikes of a plant. Anthers were cultured between May 1978 and November 1980. Groups of several hybrids were grown under identical environmental conditions, so that their responsiveness could be compared exactly, e.g. hybrid nos. 40 to 53 (see Table 3).

In experiment 2, spring barley cv. 'Dissa' was compared with its parents cv. 'Amsel' (spring type) and cv. 'Glatta' (winter type). experiment 3 comprised cvs. 'Dissa' and 'Aramir' as well as the $F_1$ hybrids 'Dissa'×'Aramir' and 'Aramir'×'Dissa'.

Anther donor plants in experiment 1 were grown in the greenhouse or in growth chambers, and out of doors, depending on the season. Temperatures varied between 8 and 12 °C up to tillering stage and between 16 and 25 °C in subsequent growth stages. During the winter, artificial light was supplied in the greenhouse (Osram lamp HQI-TS 400W/D) and growth chambers (fluorescent tubes Philips TL 215W/33RS and Sylvania Gro-Lux FR96T12/GR0235). Daylength was adjusted to 16 h at 8,000 to 10,000 lux light intensity.

Donor plants in experiments 2 and 3 were all grown under identical environmental conditions in growth chambers, at a light intensity of 18,000 to 20,000 lux (16 h daylength) and temperatures of 12 °C/5 °C (day/night). These conditions have already been proven favourable in earlier studies with cv. 'Dissa' (Foroughi-Wehr and Mix 1979).

The anther culture technique, including microscope stage, media and cultural conditions, was principally the same as described previously (Foroughi-Wehr et al. 1976).

## Results

### *Experiment 1*

The anther culture response from a random sample of 19 of the 53 hybrids in experiment 1 are summarized in Table 1. Calluses and plantlets were obtained from all of the hybrids used. Among hybrids, callus formation from 1,000 plated anthers varied between 3 and 73; the average plant production was 13.4 of which 2.0 were green (Table 2). The remaining regenerated plants lacked chlorophyll and were therefore without use for practical breeding purposes. About two thirds of the green individuals were spontaneously homozygous diploid ($2n=2x=14$), a quarter were haploid ($2n=x=7$) and a few were tetraploid ($2n=4x=28$) or aneuploid. Rates of callusing and total plant regeneration are closely related when both are based on the number of anthers plated ($r=+0.95$, Fig. 1). The two characters are, however, only weakly related, when total plant production is based on the number of calluses ($r=+0.32$). In other words, total plant produc-

**Table 1.** Anther culture response from a sample of 19 out of the 53 spring barley hybrids in experiment 1

| Hybrid | | No. | No. of anthers | No. of calluses | No. of plants | |
|---|---|---|---|---|---|---|
| | | | | | total | green |
| Villame | × 9738/3204 | 1 | 6,753 | 189 | 100 | 22 (11.6/ 3.3)[a] |
| Villame | × Trumpf | 2 | 2,181 | 95 | 49 | 14 (14.7/ 6.4) |
| Villame | × Luke | 3 | 2,472 | 47 | 24 | 9 (19.1/ 3.6) |
| Trumpf | × Villame | 4 | 3,987 | 119 | 83 | 12 (10.1/ 3.0) |
| 7512/1029 | × Villame | 5 | 4,539 | 23 | 13 | 2 ( 8.7/ 0.4) |
| 8852/2095 | × Villame | 6 | 2,478 | 34 | 22 | 9 (26.5/ 3.6) |
| E 1388 (Brückner) | × 1506 c 6434 | 14 | 5,910 | 264 | 134 | 27 (10.2/ 4.6) |
| Cornel | × E 1388 | 15 | 4,485 | 297 | 167 | 3 ( 1.0/ 0.7) |
| 8545/2922 | × E 1388 | 16 | 2,841 | 208 | 130 | 36 (17.3/12.7) |
| 8490/1520 | × E 1388 | 17 | 5,073 | 247 | 160 | 16 ( 6.5/ 3.2) |
| Trumpf | × VM 260 | 21 | 7,863 | 559 | 269 | 34 ( 6.1/ 4.3) |
| Trumpf | × 2056 b 124 | 22 | 6,804 | 216 | 82 | 6 ( 2.8/ 0.9) |
| Trumpf | × 9541/2916 | 23 | 4,254 | 237 | 129 | 14 ( 5.9/ 3.3) |
| 1474 a 2413 | × Trumpf | 24 | 5.091 | 238 | 125 | 1 ( 0.4/ 0.2) |
| Nota | × Trumpf | 25 | 5,178 | 314 | 185 | 3 ( 1.0/ 0.6) |
| Franken III | × Trumpf | 26 | 7,023 | 195 | 118 | 26 (13.3/ 3.7) |
| Carlsberg strain | × Trumpf | 27 | 5,931 | 223 | 95 | 15 ( 6.7/ 2.5) |
| Franken III | × 9557/2937 | 32 | 3,669 | 71 | 30 | 7 ( 9.9/ 2.0) |
| Nota | × 9557/2937 | 33 | 2,616 | 101 | 50 | 7 ( 6.9/ 2.7) |
| TOTAL of 53 hybrids | | | 390,775 | 10,654 | 5,217 | 797 ( 7.5/ 2.0) |

[a] in parentheses: (% of calluses / % of anthers)

**Table 2.** Summary of the anther culture response of the 53 hybrids studied in experiment 1 (390,775 anthers cultured)

| | Callus formation per 100 anthers | Plant regeneration | |
|---|---|---|---|
| | | total per 1,000 anthers | green |
| Mean | 2.9 | 13.4 | 2.0 |
| Range | 0.3–7.3 | 1.3 – 45.8 | 0 – 12.7 |
| Variance | 3.1 | 107.9 | 4.5 |
| C.V. | 0.60 | 0.71 | 0.76 |

C.V. = coefficient of variation

tion increases with rising callus formation rate, which does not necessarily mean increasing yield of total plants per single callus. Furthermore, a high frequency of total plants was not always associated with maximum green plant regeneration. For example, hybrid no. 15 yielded a total of 37.3 plants per 1,000 anthers plated, of which 0.7 were green and viable. On the other hand, from hybrid no. 6 a total of 8.9 plants per 1,000 anthers was recovered but 3.6, i.e. almost half of these, were viable. Therefore, the frequency of green regenerants is not very closely related to the rate of

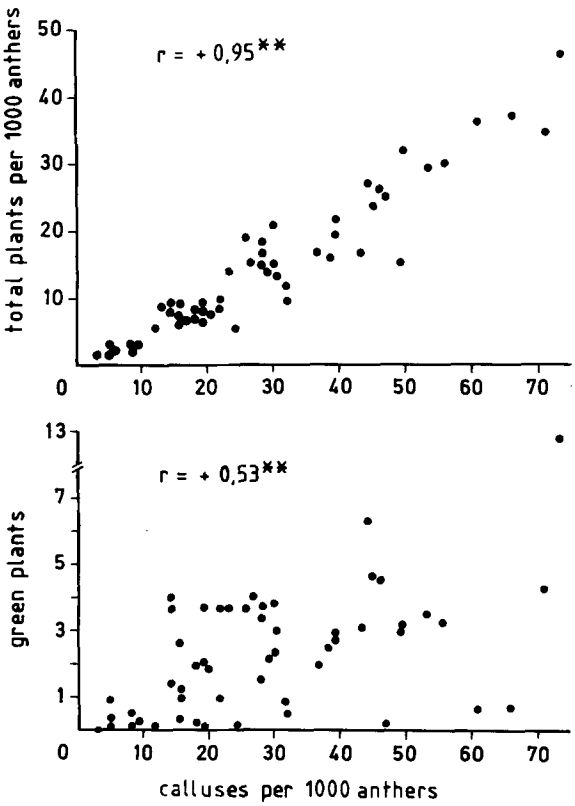


**Fig. 1.** Relationship of callus formation rate to total plant and green plant regeneration frequencies, respectively.
** significant at P = 0.01

total plant production (r = +0.61) and callus formation (r = +0.53), althoguh both correlation coefficients are statistically highly significant.

Although the results presented above indicate that genotypic differences in anther culture response are present, it must be remembered that only groups of the anther donor hybrids were grown under identical conditions. Yet, as this was the case for hybrid nos. 40 to 53 their results could be compared exactly. Spikes dissected for collecting anthers were grouped into three replications, each of which included 50 spikes. The experimental data were subsequently submitted to an analysis of variance and the results are summarized in Table 3. Highly significant variances between hybrids were found for all three characters investigated, i.e. callus, total and green plant formation. Correspondingly, the mean values for individual hybrids also differed significantly.

Differences between hybrids in anther culture responsiveness should be determined – at least partly – by their parents. In the present case 12 groups of hybrids each had one parent in common, so that parental effects could be compared. From the data in Table 3 it can be seen that the four crosses with cv. 'Hedra' as a parent are substantially poorer in anther culture than the others. Twelve hybrids in experiment 1 had cv. 'Trumpf' ('Triumph') as a common male or female parent. Seven of these – nos. 21 to 27 – were grown at the same time under identical environmental conditions, so that genotypic behaviour could be analyzed. The differences in callusing as well as plant production as presented in Table 1 are both highly significant according to a Chi-square test after Brandt & Snedecor (Sachs 1974). Hybrid no. 26 ('Franken III' × 'Trumpf') yielded the highest rate of green regenerants, although it was very poor callusing (Table 1).

The hybrids studied in experiment 1 included two reciprocal crosses (Table 4), so that maternal effects could be investigated. From a statistical analysis of the data we can say that callus formation is maternally affected in both crosses, but total plant regeneration is only influenced maternally in one of the reciprocal hybrids and green plant formation does not show significant maternal effects in both crosses (Table 4).

### *Experiment 2*

Spring barley cv. 'Dissa' has been shown in previous experiments to possess outstanding anther culture responsiveness (Foroughi-Wehr et al. 1976). Subsequently, the anther culture response of 'Dissa' and its parents 'Amsel' and 'Glatta' were studied comparatively (Foroughi-Wehr and Friedt 1981). From the data in Table 5 it can be deduced that 'Dissa' inherited most

**Table 3.** Mean values and mean squares of components of anther culture response of a group of 13 hybrids from experiment 1 (3 replications, 50 spikes per replication)

| Hybrid | No. | Mean values per 100 spikes | | |
|---|---|---|---|---|
| | | Calluses | Plants total | Plants green[a] |
| 1006 a 22 × 2056 b 12 | 40 | 80.0 | 33.3 | 10.6 |
| 2002 a 144 × 9557/2937 | 41 | 237.3 | 130.0 | 17.4 |
| 7529/1307 × 9557/2937 | 43 | 130.0 | 72.7 | 14.6 |
| 2056 b 1244 × 9557/2937 | 44 | 259.3 | 111.3 | 10.6 |
| Nordsaat 843 × 7507/1084 | 45 | 172.7 | 66.7 | 10.0 |
| 2208 c 11 × Hedra | 46 | 29.3 | 9.3 | 0.6 |
| 7507/1084 × Hedra | 47 | 18.7 | 10.7 | 0[b] |
| Hedra × 7512/1029 | 48 | 21.3 | 10.7 | 0[b] |
| Hedra × Nota | 49 | 48.0 | 22.0 | 1.4 |
| VM 260 × 9557/2937 | 50 | 82.0 | 38.7 | 0.6 |
| 9728/5325 × E 1388 | 51 | 216.7 | 87.3 | 16.0 |
| 9728/5325 × 1613 a 2534 | 52 | 136.7 | 94.0 | 19.4 |
| P 4236/76 × 1506 c 6434 | 53 | 96.7 | 55.3 | 5.4 |
| Least significant difference (LSD) | | 43.5 | 22.7 | 11.5 |
| Source of variation | d.f. | Mean squares | | |
| | | Calluses | Plants total | Plants green |
| Replications | 2 | 14.3 | 234.8 | 47.1 |
| Hybrids | 12 | 20,649.5[c] | 4,899.7[c] | 271.1[c] |
| Error | 24 | 667.2 | 180.9 | 46.6 |

[a] For statistical treatment green plant values were transformed to angles (arcsin $\sqrt{P}$, Sachs 1974)
[b] In the analysis of variance the value 0.01 was used instead of zero
[c] significant at the 0.01 level of significance

**Table 4.** Anther culture response of reciprocal hybrids in experiment 1

| Hybrid | No. | No. of anthers | No. of calluses | No. of total plants | No. of green plants |
|---|---|---|---|---|---|
| Villame × Trumpf | 2 | 2,181 | 95 | 59 | 14 |
| Trumpf × Villame | 4 | 3,987 | 119 | 83 | 12 |
| Chi-square[a] | | | 7.51 | 1.06 | 0.68 |
| P | | | 0.006 | 0.30 | 0.40 |
| Trumpf × 2056 | 22 | 6,804 | 216 | 82 | 6 |
| 2056 × Trumpf | 28 | 13,704 | 512 | 237 | 27 |
| Chi-square[a] | | | 4.02 | 3.95 | 1.65 |
| P | | | 0.05 | 0.05 | 0.20 |

[a] calculated from 2×2 Contingency tables

but not all of its good callusing ability from 'Amsel'. Thus, in addition, the outstanding green plant regeneration of 'Dissa' cannot be explained completely by inheritance from 'Amsel' (see Table 5); plausibly, recombination of favourable genes from both parents finally lead to the highly responsive genotype of 'Dissa'.

*Experiment 3*

If anther culture responsiveness is inherited then it must also be tranferable to hybrid progenies. To test this hypothesis, 'Dissa' was reciprocally crossed to the non-responsive high yielding and widely grown cultivar

**Table 5.** Anther culture response of spring barley cv. 'Dissa' and its parents 'Amsel' and 'Glatta'

| | Dissa | Amsel | Glatta | Chi-square |
|---|---|---|---|---|
| No. of anthers sown | 8,790 | 9,003 | 8,304 | |
| No. of anthers callusing | 1,019 | 471 | 210 | 625.3[a] |
| % of anthers callusing | 11.6 | 5.2 | 2.4 | |
| No. of calluses forming plants | 673 | 293 | 55 | 116.6 |
| % of calluses forming plants | 62.2 | 66.1 | 26.2 | |
| No. of calluses forming green plants | 238 | 26 | 4 | 111.9 |
| % of calluses forming green plants | 23.4 | 5.5 | 1.9 | |

[a] calculated according to Brandt & Snedecor (Sachs 1974), all values significant at P=0.001

'Aramir'. The results of anther culture of parents and hybrids are presented diagrammatically in Figure 2. It would seem that both hybrids responded like 'Dissa' in their rates of callusing and plant regeneration, but a statistical analysis of the data revealed that callus formation of 'Dissa' deviated highly significantly from that of the hybrid 'Dissa'×'Aramir' (2×2 Contingency table: $\chi^2=381.5$) and of 'Aramir'×'Dissa' ($\chi^2=107.4$). The reciprocal hybrids also differed from each other in this respect ($\chi^2=40.1$, P=0.001).

Regarding green plant production, the hybrid 'Dissa'×'Aramir' responded in a similar way to 'Dissa' itself ($\chi^2=0.1$, P=0.75), but the reciprocal hybrid produced significantly fewer green plants than 'Dissa' ($\chi^2=6.6$, P=0.01). Therefore, it can be assumed that maternal effects are acting in addition to the primary nuclear-genetically determined anther culture responsiveness, as the difference between both hybrids in their ability to regenerate green plants is statistically highly significant ($\chi^2=8.2$, P=0.004). However, it must be pointed out that this question needs further examination using other hybrids.

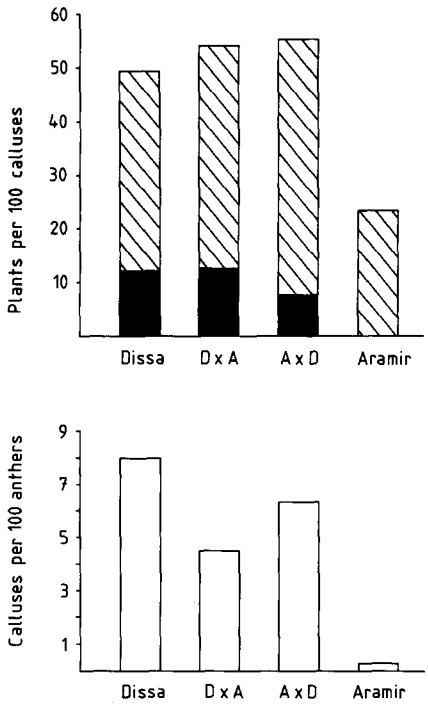


**Fig. 2.** Anther culture response of cvs. 'Dissa' and 'Aramir' and their reciprocal hybrids (D×A='Dissa'×'Aramir', A×D='Aramir'×'Dissa'), hatched=frequency of albino plants, black=frequency of green plants

## Discussion

The different responses of barley hybrid microspores cultured within the anther as reported above is in agreement with observations in such other cereals as rice (Chen and Li 1978), wheat (Picard and de Buyser 1973) and rye (Wenzel et al. 1977). In barley, the total number of regenerated plantlets depends on the frequency of callus induction; these two characters being closely related. On the other hand, the number of useful green plants cannot be predicted from the callus formation rate. Whereas one good callusing genotype may not produce green regenerants at all, another can yield many green plants. However, these genotypic differences which influence the proportions of vigorous androgenetic plants produced from barley are much less pronounced than in an outbreeding and heterozygous species like rye (Wenzel et al. 1977).

Genotypic differences in anther culture responsiveness imply that they must also be heritable. The comparison of 'Dissa' and its parents has clearly demonstrated that both callus induction and plant regeneration are inherited and are probably under the control of identical or similar nuclear genes. In potato, Wenzel and Uhrig (1981) presented data which indicated that anther culture responsiveness can be transferred from a responsive clone to breeding material which was originally non-responsive.

The outstanding green plant regeneration ability of barley cv. 'Dissa' cannot be completely explained by

simple nuclear genetic inheritance and there could well be additional nuclear or extranuclear genes controlling the production of green individuals. Their formation is also greatly influenced by environmental factors. There are many reports dealing with effects of various external influences on anther culture response, e.g. light and temperature conditions (Foroughi-Wehr and Mix 1979) and composition of culture media (Kao 1981; Xu et al. 1981), and the optimum conditions for callus induction seem to have been determined. Nevertheless, it is not clear whether different microspore genotypes also require different conditions, or if callus-stimulating modifications to the culture media could lead to increased proportions of green regenerated plants. Our own observations in barley and rye indicate that this is not the case, i.e. the proportions of green plants recovered from different callus inducing media are similar (unpublished).

In addition to environmental factors and nuclear genes there are indications that anther culture response and especially green plant formation is influenced cytoplasmically. Although the results of reciprocal crosses of 'Dissa' and 'Aramir' indicated that green plant production must be mainly affected by nuclear genes, there were significant differences between reciprocals which can be explained by maternal inheritance. When the $F_1$ donor plants had 'Dissa' as the female and 'Aramir' as the male parent, the frequency of green regenerants was significantly higher than in the reciprocal cross.

Other findings also support the opinion that there must be cytoplasmic factors influencing microspore response. It was demonstrated in androgenetic rice that albinism is accompanied by severe structural alterations of cell organelles, such as mitochondria, plastids, chloroplasts and ribosomes (Liang et al. 1978). At the biochemical level, it was shown that albino rice plants are lacking several fractions of ribosomal RNA as well as Fraction I protein which plays an important role in chlorophyll synthesis (Wang et al. 1978). These deficiencies must be caused by changes in respective genes – either nuclear or cytoplasmic.

According to the explanations above, anther culture responsiveness is probably not a simple, unique character. It seems rather to consist of several components which correspond to independent and differently inherited mechanisms, i.e.:

1) callus induction, the ability of microspores within cultured anthers to start divisions and give rise to proliferating cell accumulations,

2) callus stabilization, the preservation of complete and fully functional cells within the callus,

3) plantlet induction, the ability of cells within calluses to give rise to embryos and plants, and

4) green plant formation, the production of fully functional green haploid and doubled haploid plants.

At each step there are genetic as well epigenetic factors affecting the culture system. In other words, each genotype is subjected to environmental influences, and it can be assumed that responsive genotypes do not require highly specific culture conditions for growth.

There are two principal ways of improving anther culture response, namely the adjustment of culture conditions and the genetic improvement of anther donor material. Because of the dominating effects of genetic factors demonstrated above, the latter way is at the present time more useful for practical application. The transfer of anther culture responsiveness to non-responsive breeding material appears easier than testing numerous culture conditions when there is a possibility that each microspore might require its own specific medium.

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