Theor. Appl. Genet. 55, 257-262 (1979)

Biochemical Selection of Immature, Haploid Embryos of *Zea mays* L.

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Summary. A method was devised for the biochemical selection of immature, haploid *Zea mays* embryos using *Adh1*$^-$ and either the Stock 6 or indeterminate gametophyte (*ig* in W23) high haploid-inducing systems. Haploid (*Adh1*$^-$) embryos survived exposure to levels of allyl alcohol which killed diploid (*Adh1*$^+$/*Adh1*$^-$) embryos. Of the total surviving embryos which were examined cytologically 15% (using *ig*) and 22% (using Stock 6) were haploid. In two experiments with Stock 6, 100% of the surviving embryos were haploid. To obtain maximum effectiveness of Stock 6 and *ig*, *Adh1*$^-$ was transferred to stock 6 and W23 backgrounds. Immature, haploid embryos are being used to develop haploid, morphogenic tissue cultures of *Zea mays*.

Key words: Biochemical selection – *Zea mays* – Haploid

Introduction

The development of somatic cell genetics in *Zea mays* requires a source of cells which are both haploid and totipotent. Maize haploids may be detected at their spontaneous frequency using recessive markers such as *yg2*. They may be more readily obtained using Stock 6 (Coe 1959) or the indeterminate gametophyte (*ig*) mutant (Kermicle 1969) to enhance the frequency (to ca. 3%) of gynogenetic and androgenetic haploids, respectively. In each case, the markers used to detect haploids are chosen for screening at the mature seed or seedling stage. Tissue cultures have been established from haploid plants so derived, and the haploid state shown to be relatively constant (Dhaliwal and King 1979). However, the totipotency of somatic cells from mature maize organs has never been demonstrated and such haploid tissue cultures do not regenerate plants. In contrast, Green and Phillips (1975) and Dunstan et al. (1978) have shown that cells in the scutellum of immature cereal embryos are totipotent and that tissue cultures established from the scutellum of certain varieties repeatedly regenerate plants. In this paper we report on the use of the alcohol dehydrogenase null maize mutant (*Adh1*$^-$) (Schwartz 1969) as a biochemical marker for the detection and recovery of immature, haploid embryos a few days post-pollination. Such embryos might then be used to initiate morphogenic, haploid tissue cultures.

Allyl alcohol has been used to select mutant pollen lacking alcohol dehydrogenase (isoenzyme 1) (ADH1) activity (Schwartz and Osterman 1976; Cheng and Freeling 1976). The mutant pollen do not convert allyl alcohol to the toxic acrylaldehyde and are thus more resistant to allyl alcohol exposure. ADH1 is the only ADH isozyme active in the pollen grain and it accounts for most of the activity in the scutellum of immature embryos (Scandalios and Felder 1971[1]). In the selection system described in this paper (diagram in Fig. 1) homozygous *Adh1*$^-$ mutant plants are used as a parent in crosses with haploid-inducing genetic stocks to select for immature haploid embryos after allyl alcohol exposure. Haploid embryos (*Adh1*$^-$) survive the exposure whereas diploid embryos (*Adh1*$^+$/*Adh*$^-$) are killed.

Material and Methods

Seed stocks

These were obtained as follows: *Adh1*$^-$ from Dr. D. Schwartz (University of Indiana), Stock 6 from Dr. E. Coe (University of Missouri) and *ig* in W23 from Dr. J. Kermicle (University of Wisconsin) and Dr. G. Gavazzi (University of Milan).

1 *ADH1* of Schwartz and of Freeling corresponds to *ADH2* of Scandalios

0040-5752/79/0055/0257/$ 1.20

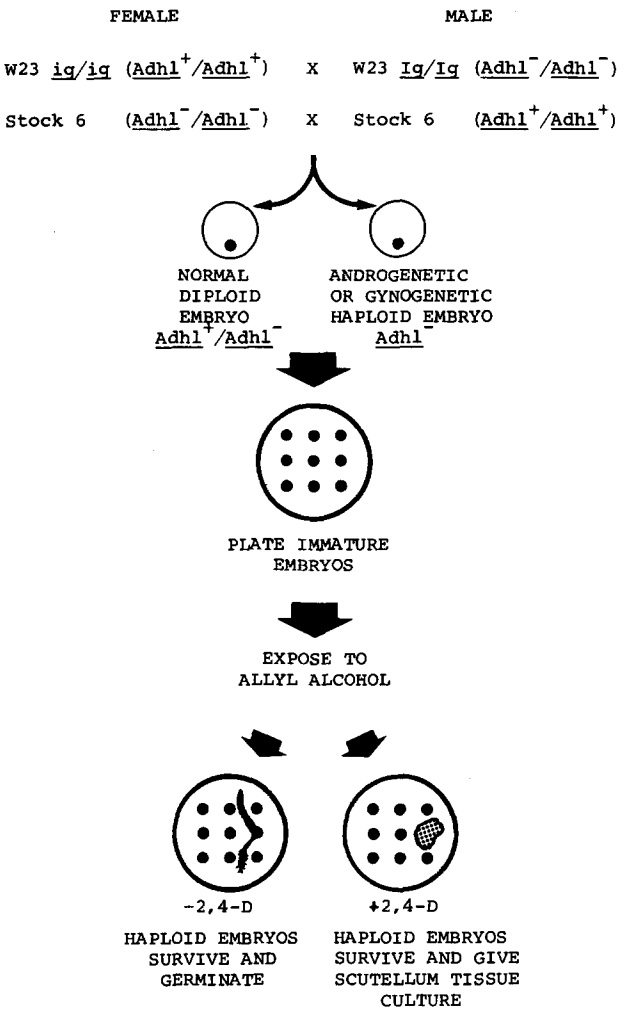

Fig. 1. A diagrammatic summary of the method used to isolate immature, haploid embryos using *Adh1*⁻ as a biochemical marker

Embryo Plating

Developing caryopses containing embryos 1.5-2.0 mm long were detached individually from the cob, sterilized in sodium hypochlorite (2.5% plus a few drops of detergent per litre, 20 min) and washed three times with sterile water. Embryos were dissected out and plated onto modified MS medium with/without 2,4-D (Green and Phillips 1975) in 55 mm sterile, plastic petri-dishes with the scutellum or embryonic axis in contact with the medium respectively.

Allyl Alcohol Treatment

Each petri-dish containing ca. 20 plated embryos was placed without a lid into a sterile, airtight container having a volume of 300 ml. An appropriate volume of allyl alcohol diluted in methanol was pipetted with a micro-syringe onto the wall of the container and the lid immediately replaced. After a specified time the dish was removed and the embryos were incubated either in situ or after transfer to fresh medium. Dishes were sealed with parafilm and incubated at 28°C with a 16 h photoperiod. Methanol alone did not inhibit germination at the maximum concentration routinely used.

Germination

Embryo germination was scored after 3-4 days. Germinating embryos (putative haploids) were transferred to fresh medium and incubated under the same conditions to await root development.

Chromosome Counts

Root tips (10-15 mm) were pretreated with a saturated aqueous solution of α-bromonaphthalene (3.5 h), fixed in ethanol:acetic acid (3:1 v/v, 12 h), hydrolysed in HCl (1.0 N, 60°C for 12 min), washed and softened in pectinase (Fluka, 5.0% w/v, 1.5 h) and stained in propionic-orcein (1.0% w/v).

Transfer of Adh1⁻ to Stock 6 and W23

In 1977 crosses were begun between Stock 6 and W23 (as female) and homozygous *Adh1*⁻ stock. Pollen of F_1 plants was treated with allyl alcohol prior to selfing or backcrossing with parental stocks to eliminate *Adh1*⁺ pollen (Cheng and Freeling 1976). In order to shorten the generation time and to further eliminate *Adh1*⁺/*Adh1*⁻ progeny, immature embryos from selfed F_1 were dissected out, plated and exposed to allyl alcohol as described above. Surviving embryos were cultured further in vitro before being potted and eventually transferred to the field. The genotype of flowering plants was confirmed using an ADH-specific cytochemical pollen staining technique (Freeling and Brown 1975). The same procedures were used during BC_1 selfing. The F_2 plants (homozygous *Adh1*⁻ in 50% Stock 6 or W23 background) and the BC_1 plants (heterozygous *Adh1*⁺/*Adh1*⁻ in 75% Stock 6 or W23 background) were used as female (Stock 6) or male (W23) in crosses with Stock 6 or W23 (*ig/ig*) respectively in the haploid selection experiments.

Acetaldehyde Treatments

Filter-sterilized acetaldehyde solutions were added to autoclaved agar MS medium (plus 2,4-D at 9.0×10^{-6}M) at 40°C. The dose-response effect was determined using replicate 50-100 mg pieces of established *Adh1*⁺/*Adh1*⁻ and *Adh1*⁻/*Adh1*⁻ cultures. Response was measured as relative increase in fresh weight after 28 days incubation at 28°C.

Results

Allyl Alcohol Treatment

The optimum allyl alcohol exposure which discriminated between wild type (*Adh1*⁺/*Adh1*⁺ or *Adh1*⁺/*Adh1*⁻) and mutant (*Adh1*⁻/*Adh1*⁻) embryos was initially determined using the heterozygous diploid embryos produced from the cross W23 *ig* × *Adh1*⁻/*Adh1*⁻, and embryos from selfed homozygous *Adh1*⁻ stock. Wild type and mutant embryos were plated on the same dishes and exposed for 10 min to varying concentrations of allyl alcohol. The embryos were

further incubated on the exposed dishes and scored for germination. A treatment of 12.5 μM allyl alcohol for 10 min was found to be optimum (Table 1). In later experiments, heterozygous F_1 embryos from [F_2 (Stock 6 × *Adh1*⁻) *Adh1*⁻/*Adh1*⁻] × Stock 6 were used as wild type controls. The optimum selective exposure was again found to be 10-12.5 μM allyl alcohol for 10 min (Fig. 2A,B). However, when transferred to fresh medium immediately after exposure at the selective level, wild type embryos recovered and germinated normally. Unexposed embryos died when plated amongst exposed embryos on exposed medium but germinated normally when plated together with exposed embryos (at 10 × the normal selective level) on unexposed medium. These results rule out cross inhibition of *Adh1*⁻ embryos by acrylaldehyde leaking from wild type embryos but clearly show the absorption of allyl alcohol by the agar medium. To avoid the danger of delayed inhibition of haploid, *Adh1*⁻ embryos due to the action of ADH2 isozyme on allyl alcohol persisting in the incubation medium, a high selective exposure was found (125 μM for 10 min) which allowed immediate transfer of all embryos to fresh incubation medium (Fig. 2C,D).

Table 1. Sensitivity of immature *Zea mays* embryos to allyl alcohol vapour

Allyl alcohol concentration x 10^{-6} M	Response	
	Adh1⁻/*Adh1*⁻	*Adh1*⁺/*Adh1*⁻
0.0	+++	+++
2.5	+++	++
6.3	+++	+
12.5	+++	–
25.0	–	–

All exposures were for 10 min. '+' indicates tissue culture growth from scutellum. The heterozygous embryos were derived from the cross W23 *ig* × *Adh1*⁻/*Adh1*⁻

The effectiveness of this selective exposure in distinguishing wild type embryos from homozygous mutant embryos of the same age, size and genetic background was tested using the progeny of the selfed BC1 plants during the transfer of *Adh1*⁻ to Stock 6 and W23 (Materials and Methods). From normal selfing, 25% homozygous *Adh1*⁻ plants were expected. Following treatment of pollen with allyl alcohol prior to selfing, 50% homozygous *Adh1*⁻ plants were expected. Embryos from the selfed plants were dissected 20-25 days after pollination, plated onto MS medium, exposed to allyl alcohol at 125 μM for 10 min and transferred immediately to fresh medium. The expected and observed frequencies of surviving embryos are given in Table 2.

Selection of Stock 6-Induced (Gynogenetic) Haploid Embryos

In the summer of 1977, initial crosses were made between homozygous *Adh1*⁻ stock (as female) and Stock 6, and a small number of embryos were unsuccessfully screened with allyl alcohol. (Table 3A). At the same time a crossing

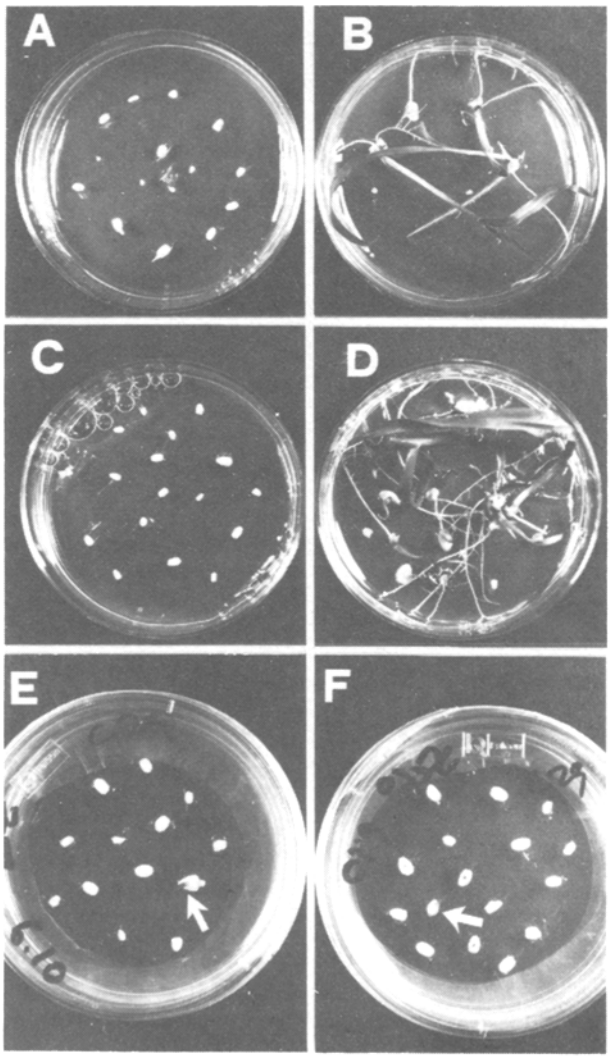

Fig. 2A-F. Development of immature embryos of *Zea mays* in vitro following exposure to allyl alcohol vapour. Embryos were dissected 20-25 days post-pollination and plated on MS medium −2,4-D. A Embryos from the cross F_2 (Stock 6 × *Adh1*⁻) *Adh1*⁻/*Adh1*⁻ × Stock 6 exposed to 10 μM allyl alcohol for 10 min and incubated on the exposed plates. B Homozygous *Adh1*⁻ embryos, treated as in (A) above. C Embryos from the cross F_2 (Stock 6 × *Adh1*⁻) × Stock 6 exposed to 125 μM allyl alcohol for 10 min and transferred immediately to fresh medium for germination. D Homozygous *Adh1*⁻ embryos treated as in (C) above. E and F Embryos from the cross F_2 (Stock 6 × *Adh1*⁻) *Adh1*⁻/*Adh1*⁻ × Stock 6 exposed as in (C) and (D) above. Arrows indicate germinating embryos which were isolated, grown up and later confirmed as haploid by chromosome counts

programme was begun to transfer *Adh1*$^-$ to Stock 6 background (Materials and Methods).

In 1978 further crosses were made between F_2 (Stock 6 × *Adh1*$^-$) (homozygous for *Adh1*$^-$) as female and Stock 6, and the progeny screened with allyl alcohol (Table 3B). From 1717 embryos plated, 50 embryos survived. Only 40% of the surviving embryos could be examined cytologically due to poor root formation; 5 haploids were recovered. In two of the experiments (No. 6 and 10, Table 3B), all surviving and rooting embryos were haploid (see also Fig. 2E,F). In one further experiment with heterozygous F_2 plants (Table 3C), 2 from 115 embryos survived, one of which rooted and proved to be haploid, giving a total of 6 haploid plants from 1832 embryos screened. In two later experiments with heterozygous BC1[Stock 6 × (Stock 6 × *Adh1*$^-$)] plants no haploids were recovered from 239 embryos, 11 of which had survived and 7 had produced roots (Table 3D).

Table 2. The effect of allyl alcohol exposure on immature embryos derived by selfing *Adh1*$^+$/*Adh1*$^-$ plants with or without prior treatment of pollen with allyl alcohol

Selfed parent[a]	Pollen treatment	Number of embryos		
		screened	expected to germinate	germinated
1) Stk6 × (Stk6 × *Adh1*$^-$)	allyl alcohol	74	37	35
2) Stk6 × (Stk6 × *Adh1*$^-$)	none	188	47	49
3) W23 × (W23 × *Adh1*$^-$)	allyl alcohol	132	66	64

Allyl alcohol exposure was 125 μM for 10 min followed by immediate transfer of embryos to fresh medium

[a] Prior to the first backcross the pollen of the F_1 (i.e. Stk6 × *Adh1*$^-$ or W23 × *Adh1*$^-$) was treated with allyl alcohol; thus all backcross 1 plants should be heterozygous for *Adh1*

Table 3. Selection of immature, haploid *Zea mays* embryos by allyl alcohol treatment after crosses involving Stock 6 and *Adh1*$^-$ stocks

Experiment	No.	Cross	*Adh1* genotype of female	Number of embryos			Number of haploids
				plated	germinated	counted	
A							
1977	1	*Adh1*$^-$ × Stk6	*Adh1*$^-$/*Adh1*$^-$	200	0	0	0
B							
1978	2	F_2 (Stk6 × *Adh1*$^-$) × Stk6	"	226	4	1	1
	3	" "	"	156	0	0	0
	4	" "	"	187	0	0	0
	5	" "	"	350	32	12	0
	6	" "	"	200	2	2	2
	7	" "	"	177	3	0	0
	8	" "	"	165	3	2	0
	9	" "	"	120	4	1	0
	10	" "	"	136	2	2	2
C							
1978	11	" "	*Adh1*$^+$/*Adh1*$^-$	115	2	1	1
D							
1978	12	Stk6 × (Stk6 × *Adh1*$^-$) × Stk6	*Adh1*$^+$/*Adh1*$^-$	105	3	1	0
	13	" "	"	134	8	6	0
Totals				2271	63	28	6

Allyl alcohol exposure was 12.5 μM for 10 min followed by incubation on exposed medium (Expt. No. A1 and B2) or 125 μM for 10 min and incubation on fresh medium (all other experiments)

Selection of ig-Induced (Androgenetic) Haploid Embryos

In 1977 crosses were made between *igig* or *Igig* (W23) as female and homozygous *Adh1*$^-$ stock. The intention in these early experiments was to obtain tissue cultures directly from the surviving embryos. Thus, 1133 embryos were plated onto MS medium plus 2,4-dichlorophenoxy acetic acid (9.0×10^{-6}M), exposed to allyl alcohol at 12.5 μM for 10 min and left on the exposed medium for germination. Scutellum tissue cultures were induced on 21 embryos. After 3 passages the chromosome numbers of the surviving cultures were checked: in three cultures a mixture of haploid and diploid metaphases was found, all other cultures were diploid. A further biochemical test was devised to check for doubled haploids amongst the surviving cultures: the growth of wild type (*Adh1*$^+$) cultures was enhanced by acetaldehyde up to 10^{-1}M, whilst homozygous *Adh1*$^-$ cultures were inhibited at 10^{-6}M acetaldehyde. All 21 surviving cultures were screened at 10^{-3}M acetaldehyde: 8 were inhibited, including the mixed haploid/diploid lines.

In 1978 *Adh1*$^-$ was also introduced into W23 background (see Materials and Methods). F_2 plants homozygous for *Adh1*$^-$ (in 50% W23 background) and BC1 plants heterozygous for *Adh1*$^-$ (75% W23 background) were then used, after allyl alcohol pollen treatment, to pollinate W23 *igig* (male sterile) or *Igig* (fertile) plants. To date 600 embryos have been screened by exposure to 125 μM for 10 min and transfer to fresh medium without 2,4-D. Twelve out of 14 survivors were examined, all were diploid.

Discussion

Allyl alcohol is known to be a powerful selective agent discriminating between wild type and *Adh1*$^-$ pollen grains (Schwartz and Osterman 1976). The results in Table 2 show a very efficient selection of the ADH$^-$ condition at the immature embryo level. Allyl alcohol can also be used to select homozygous *Adh1*$^-$ amongst dry seeds (Dhaliwal and King, unpublished observation). The selection procedure reported here was determined using diploid, homozygous *Adh1*$^-$ immature embryos on the assumption that the sensitivity of haploid, *Adh1*$^-$ embryos would be the same. It is not yet possible to completely verify this assumption.

In the 1977 experiments with Stock 6 (Table 3A), the failure to recover haploids may be due to the absence of Stock 6 background in the maternal parent. Coe (1959) and Sarkar and Coe (1966) reported an increase in the frequency of maternal haploids as the proportion of Stock 6 background increased in the male and female parents. With Stock 6 as female, the use of Stock 6 as male increases the haploid frequency by several fold. It is not clear whether maternal haploids are induced when only the male is Stock 6. In view of this uncertainty and because Stock 6 induces maternal haploids we transferred *Adh1*$^-$ to Stock 6 background. The F_1 hybrid (Stock 6 $\times$ *Adh1*$^-$) was selfed and backcrossed with Stock 6 after allyl alcohol pollen treatment. Ca. 50% of the F_2 plants were homozygous for *Adh1*$^-$ whilst all first generation backcross plants were heterozygous as determined by the ADH specific pollen staining assay.

In the majority of experiments in 1978, homozygous F_2 plants were used as female and pollinated with Stock 6. Immature haploid embryos were recovered from 4 out of 10 F_2 plants (Table 3). In one experiment (No. 5, Table 3B) ca. 10% of the plated embryos germinated but no haploids were recovered. This was probably the result of accidental selfing as the maternal plants were not detasseled, and as the frequency of parthenogenetic diploids in Stock 6 was reported to be only ca. 0.01% (Sarkar and Coe 1971). Despite this anomalous experiment, a total of 28 embryos (44% of all germinating embryos) was examined cytologically, of which 6 (22%) proved to be haploid.

The failure to recover haploids from many of the F_2 plants may simply be due to the relatively small number of embryos screened and/or to the segregation of factors responsible for haploid induction in Stock 6. From the limited size of a segregating progeny, Coe (1959) suggested the existence of a two-factor system for haploid induction in Stock 6. In our later experiments two BC1 plants heterozygous for *Adh1*$^-$ gave no haploids (Table 3D) despite the increased proportion of Stock 6 background (75%) compared to the F_2 plants (50%, Table 3B). This may be due both to the low number of embryos screened and the heterozygosity of the female parent. On the basis of the results of Sarkar and Coe (1966), we expect to completely recover the haploid-inducing ability of Stock 6 in the BC2 generation. Thus, the haploid frequency (in our experiments a maximum of 1.0-1.5%, experiments No. 6 and 10, Table 3B) should increase to $>$ 3%.

In the 1977 experiments with the *ig* system, homozygous *Adh1*$^-$ stock was used as the pollen parent in crosses with W23 homozygous or heterozygous for *ig*. Scutellum cultures were induced from 21 out of 1133 plated embryos. Three of the 21 cultures were confirmed as haploid from chromosome counts and a further 5 cultures were considered putative doubled haploids from their sensitivity to acetyldehyde. This means that 38% of the surviving embryos were haploid but that the maximum probable haploid frequency in these experiments was only 0.7%. This compares unfavourably with the frequency (ca. 3%) reported by Kermicle (1969) for the *ig* system. However, Kermicle (1969) reported a drastic re-

duction in haploids when W23 background was removed from the pollen parent. A similar problem is reported by corn breeders using *ig* for gamete selection programmes or extraction of instant inbreds. We therefore transferred *Adh1*$^-$ to W23, and F_2 plants (homozygous *Adh1*$^-$ in 50% W23) as well as BC1 plants (75% W23) were used as pollen parents in 1978. Those survivors in 1978 (14 from 600) which were examined were all diploids. It is possible that some of the survivors are androgenetic diploids (Kermicle 1969; Greenblatt and Brock 1967) and will be checked further at maturity. The exact constitution of the *ig* stock used in the latter experiments is in some doubt; it did not, for example, show the expected pleiotropic effects of *ig* in W23 such as twin embryos.

Kermicle (1969) reported a 4-5 fold increase in maternal haploids with *ig* as female compared to W23 *Ig*. Furthermore, *Igig* as male gave more maternal haploids than *IgIg*. It is obvious that the haploid-inducing ability of Stock 6 is due to genes other than *ig*. Thus, by introducing *ig* into Stock 6 along with *Adh1*$^-$ it may be possible to increase maternal haploid frequency by 4-5 fold, i.e. to 10-15%.

Despite the high selectivity of allyl alcohol shown in the selection tests and in several of the haploid recovery experiments, *Adh1*$^-$ may not be the ideal mutant for use in selecting immature haploid embryos. There is some indication that absence of ADH activity reduces the vigour of cultures and plantlets under the culture conditions used. Green and Donovan (1978) have used the *R-nj* pigment marker in the *ig* stock with partial success but this method is limited by the presence of interfering pigment genes in the pollen parent and variable expression of *R-nj* in the scutellum of immature embryos. Preliminary experiments in our laboratory suggest that the recessive fluorescence mutants *Bf* and *bz* might make excellent selection markers which would allow haploids to be picked out simply by scanning plated embryos with a long-wavelength UV lamp.

The aim of this programme of haploid selection is to provide immature haploid embryos of *Zea mays* from which morphogenic, haploid tissue cultures may be derived or from which haploid totipotent protoplasts may be isolated. In the early experiments with W23 *ig* reported here, cultures were initiated immediately from surviving embryos. Because of an apparent problem of spontaneous haploid doubling, embryos surviving in later experiments were allowed to germinate to provide unequivocal evidence of haploid frequency. Upon completion of the *Adh1*$^-$ transfers described above, work on culture initiation will continue.

Acknowledgement

The authors are grateful to Alice Frischknecht and Gabriele Persy for skilled technical assistance and to colleagues in the Friedrich Miescher-Institut for helpful discussion.

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Received April 20, 1979
Communicated by G. Melchers

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