M. Feldman

Sonderdruck aus „Zeitschrift für Pflanzenzüchtung“
[Z. Pflanzenzüchtg. 82, 36—44, 1979]

Verlag Paul Parey, Berlin und Hamburg

Max-Planck-Institut für Züchtungsforschung (Erwin-Baur-Institut)
Abteilung Straub, Köln-Vogelsang (Germany F.R.)

Cytoembryological Studies on the Process of Fertilization and the Development of Haploid Embryos of *Triticum aestivum* L. (2n = 42) after Crossing with *Hordeum bulbosum* (2n = 14)

M. Zenkteler and J. Straub

Z. Pflanzenzüchtg. **82**, 36—44 (1979)

ISSN 0044-3298 / ASTM-Coden: ZEPZAD

Max-Planck-Institut für Züchtungsforschung (Erwin-Baur-Institut)
Abteilung Straub, Köln-Vogelsang (Germany F.R.)

Cytoembryological Studies on the Process of Fertilization and the Development of Haploid Embryos of *Triticum aestivum* L. (2n = 42) after Crossing with *Hordeum bulbosum* (2n = 14)

M. Zenkteler and J. Straub

With 2 figures and one table

Received February 17, 1978 / Accepted February 18, 1978

In the past years, a number of papers appeared dealing with the production of haploids in barley after pollination by *Hordeum bulbosum* (Lange 1969, 1971, Kasha and Kao 1970, Subrahmanyam and Kasha 1973, Kasha 1974, Davies 1974, Subrahmanyam 1977). Studies were also carried out to determine whether this technique of haploid production could be extended to other species. Using this method Barclay (1975) obtained haploid plants from crossing *Triticum aestivum* (2n = 42) cv. 'Chinese Spring' with *Hordeum bulbosum* (2n = 14 or 28), and Fedak (1976) reported on the development of barley × rye crosses. All the available reports suggested that haploids arose through a process of elimination of the male partner chromosomes.

At present, the use of *Hordeum bulbosum* for haploid production in crosses with barley and wheat seems to be more effective for practical purposes than the method of anther culture. However, there is a lack of information on the possibility of obtaining haploids in crosses with wheat varieties other than 'Chinese Spring', and no data are available on the process of fertilization in those crosses. The following report was undertaken to reveal: firstly, the process of fertilization in *Triticum aestivum* cvs. 'Chinese Spring', 'Bali', and 'Janus' after crossing with *Hordeum bulbosum*; secondly, the development of embryos and endosperm in 'Chinese Spring' after crossing with *H. bulbosum*.

Material and Methods

Plants of *Triticum aestivum* cvs. 'Chinese Spring', 'Janus', and 'Bali' were maintained throughout their growth period in a greenhouse at 16 °C. Florets in spikes were emasculated 1—2 days prior to anthesis and later on the spikes were enclosed in bags to prevent pollination. Subsequently the plants were transferred to a warm greenhouse with temperatures of 20 to 25 °C. On the second or third day after emasculation bags were removed while the stigmas were handpollinated with fresh pollen of *H. bulbosum* grown under field conditions. After pollination, the bags were immediately replaced. Plants which grew in the cool greenhouse as well as those which after pollination were transferred to the warm one were both maintained at natural daylength during the period of June—September 1977. 2102 pistils of 'Bali', 2100 of 'Janus', and 2110 of 'Chinese Spring' were jointly pollinated. Ovaries for fixing were taken at the following time intervals (in hours): 0.5—1, 1, 2, 4, 6, 12, 24, 48, 72, 96, 120, 168, and 216. The material was fixed in 1 : 3 acetic alcohol for 24 hours, then dehydrated in series of alcohols and embedded in paraffin. After dehydration a part of the material was transferred to aceton and embedded in Paraplast. Sections were cut mostly longitudinally in the dorsi-ventral plane at 15—18 μm, mounted, and stained using Heidenhain's hematoxylin with fast green counterstain or with crystal violet and orange G counterstain. 727 ovaries were jointly sectioned and analyzed. The numbers of examined ovaries of 3 varieties fixed after 13 intervals following pollination are listed in *Table 1*.

The pollen tube growth in stigmas was analyzed by using cotton blue staining described by D'Souza (1972).

10—14 days after pollination, the induced seeds were surface sterilized with mercuric chloride during 2—5 minutes and washed 3 times with sterile water. The embryos were dissected and placed on the culture medium described by Gamborg, Miller and Ojima (1968), omitting, however, 2,4-D and adding 7.0 g of bactoagar per liter of solution. The embryos were incubated at 22—25 °C in a light chamber. The seedlings at the 2—3 leaf stage were removed from the vials and potted in soil.

Chromosome numbers of plants were determined by fixing root tips in acetic-ethanol, washed with distilled water, hydrolysed in 1 N HCl at 60 °C for 15 minutes and stained in acetocarmine.

Tab. 1 Number of analyzed ovules

Varieties	Time (in hours) after pollination												
	0.5 to 1	1	2	4	6	12	24	48	72	96	120	168	216
'Bali'	22	18	20	20	15	18	16	20	15	18	18	10	—
'Janus'	26	20	20	12	22	10	20	26	28	20	15	12	—
'Chinese Spring'	20	20	22	20	15	15	20	24	26	22	30	24	28
	68	58	62	52	52	43	56	70	69	60	63	46	28

Results

The pollen grains of *Hordeum bulbosum* germinated almost immediately after being attached to the stigma of *Triticum aestivum*. Fifteen minutes after pollination a great number of pollen tubes passed through the lobes of the stigma and grew in the conducting tissue of the style (*Figs. 1A* and *B*). Germinating and growing pollen tubes were observed in the stigmas and styles of all 3 varieties and practically all of them grew in the direction to the ovary. Usually, under the conditions present in the greenhouse, the pollen tubes reached the embryo sacs in 30—40 minutes, but only one tube entered the

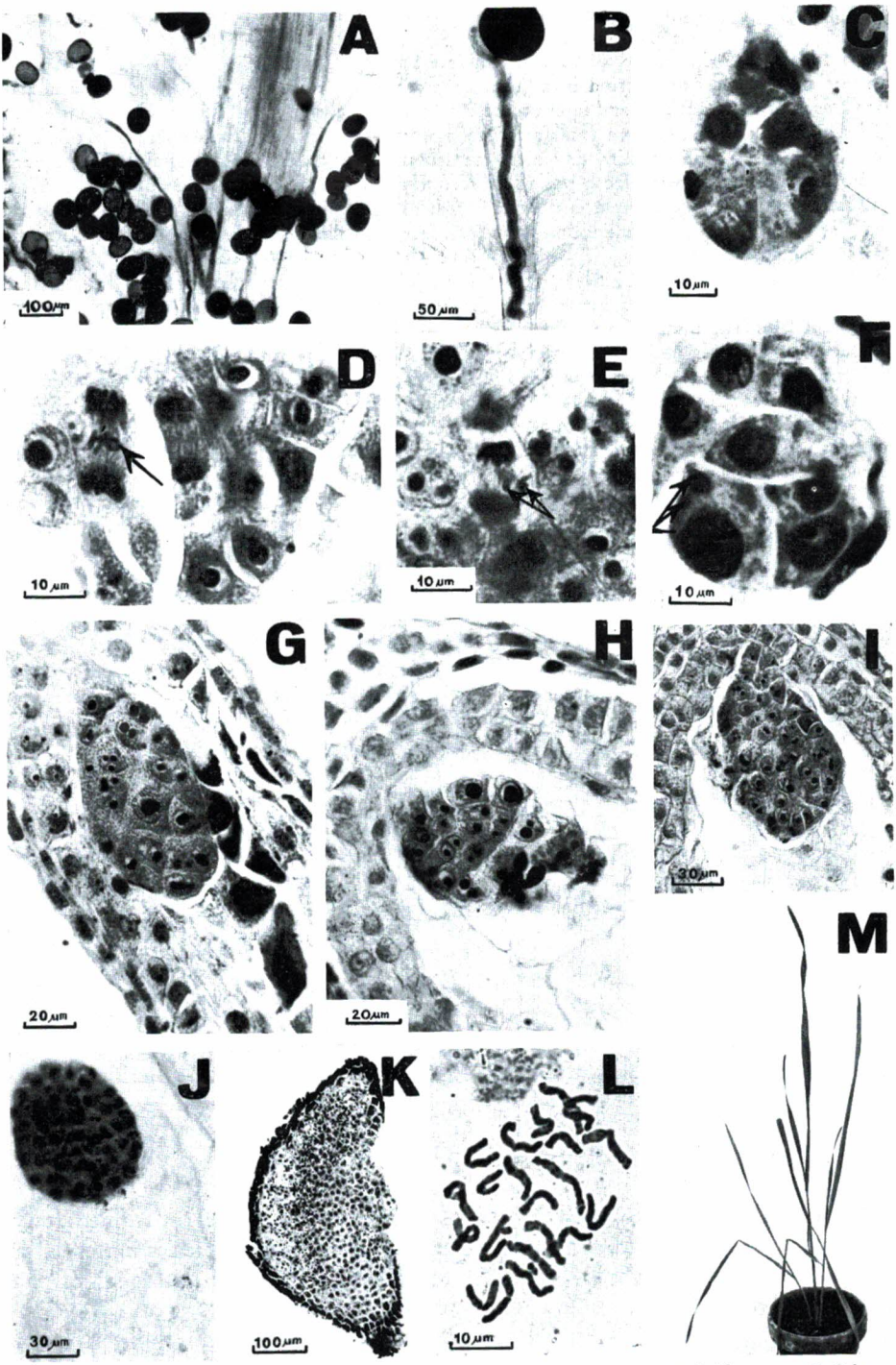

Fig. 1 A Growth of pollen tubes in the cross 'Chinese Spring' × *H. bulbosum*; B Stigma hair with one pollen tube growing into the direction of the ovary, 'Bali' × *H. bulbosum*; C—M 'Chinese Spring' × *H. bulbosum*: C Small proembryo inside the ovule, endosperm

micropylar end of the embryo sac. After the penetration into the embryo sac the pollen tube discharged its content in one synergid. In some embryo sacs, however, both synergids were destroyed which shows that also the second synergid was affected by the entrance of the tube. The first male gamete came into contact with the egg cell about 40—60 minutes after entrance, while the second one was already fusing with the nucleus of the central cell. In cases when the polar nuclei were drawn together but not fused the male gamete got into contact with one of them. Double fertilization occurred in all three varieties. In the nuclei of egg cells as well as in polar nuclei, two or more nucleoli were present 4 hours after pollination. From this moment onward the developmental process were different in 'Chinese Spring' and in 'Bali' and 'Janus'. Therefore, the 2 groups of plants will be described separately.

Triticum aestivum cv. 'Chinese Spring' × ***Hordeum bulbosum***

The zygote prepared itself for division until the period of 36 hours after pollination, and then probably started to divide a few hours later. This is only an indication, as in our observations we were not able to find the exact division moment of the zygotes. The youngest stages of the developing proembryos were found on the slides only 3 days after pollination (*Fig. 1 C*). It was also difficult to ascertain the exact duration of the dormancy period of the fertilized central cell. Usually, at the third day after pollination, when a small proembryo was present, several endosperm nuclei were also observed inside the embryo sac cavity. However, only very few nuclei were irregularly scattered in a poor cytoplasm in comparison to the control material with embryo sacs of self pollinated plants in which at that time the endosperm was rich and well developed (*Fig. 1 G*). In some embryo sacs a few endosperm nuclei were visible without the presence of embryos. It appears that in these cases the egg cells of zygotes were degenerated and only the endosperm nuclei were able to divide. Also embryo sacs were found in which small proembryos had developed, without endosperm as shown in *Figure 1 C*. However, these cases were very rare and usually paralleled the development of proembryos, in which also a poor endosperm was discernible.

In 3—5 day old embryos different cells showed differing abnormalities during mitosis. The most common ones were chromosome elimination which was visible during the anaphase-telophase stages (*Figs. 1 D* and *E*) as well as the formation of micronuclei and condensed chromatin bodies. In some cells more than one nucleus was present, and additional micronuclei of various sizes were frequently found (*Fig. 1 F*). In embryos analyzed 7—9 days after pollination the cells often degenerated in various regions of the embryo, and it was easy to distinguish them as the nuclei were represented in the form of condensed bodies with the cell wall usually collapsed (*Fig. 1 H*).

---

not present, 3 days after pollination (d.a.p.); D, E Chromosome elimination (arrows) in a dividing embryo cell, 5 d.a.p.; F Additional micronuclei (arrow) in an embryo cell, 4 d.a.p.; G Developing embryo with endosperm in a control ('Chinese Spring' self pollinated), 3 d.a.p.; H Degenerating embryo inside ovule, 7 d.a.p.; I, J Embryo inside ovules 7 and 9 d.a.p., respectively, endosperm not developed; K Embryo isolated from ovule, 14 d.a.p.; L 21 chromosomes in a haploid wheat plant; M Haploid plant of 'Chinese Spring', 6 weeks after transfer of seedling into soil

As the age of embryos advanced, the size of embryos incraesed although there were wide variations in the size and shape. Exact chromosome numbers were difficult to determine in embryos from permanent slides. When metaphase plates were present, the cells contained fewer than 28 chromosomes. In ovules examined 7, 9, and 12 days after pollination, the observed embryos were of different stages and sizes. The most typical stages were the ones shown in *Figures 11* and *J*.

In all caryopses examined 9 days after pollination no endosperm was ever found. The only indications of an earlier formed endosperm were residues of degenerating nuclei mixed together with the liquid of the embryo sac.

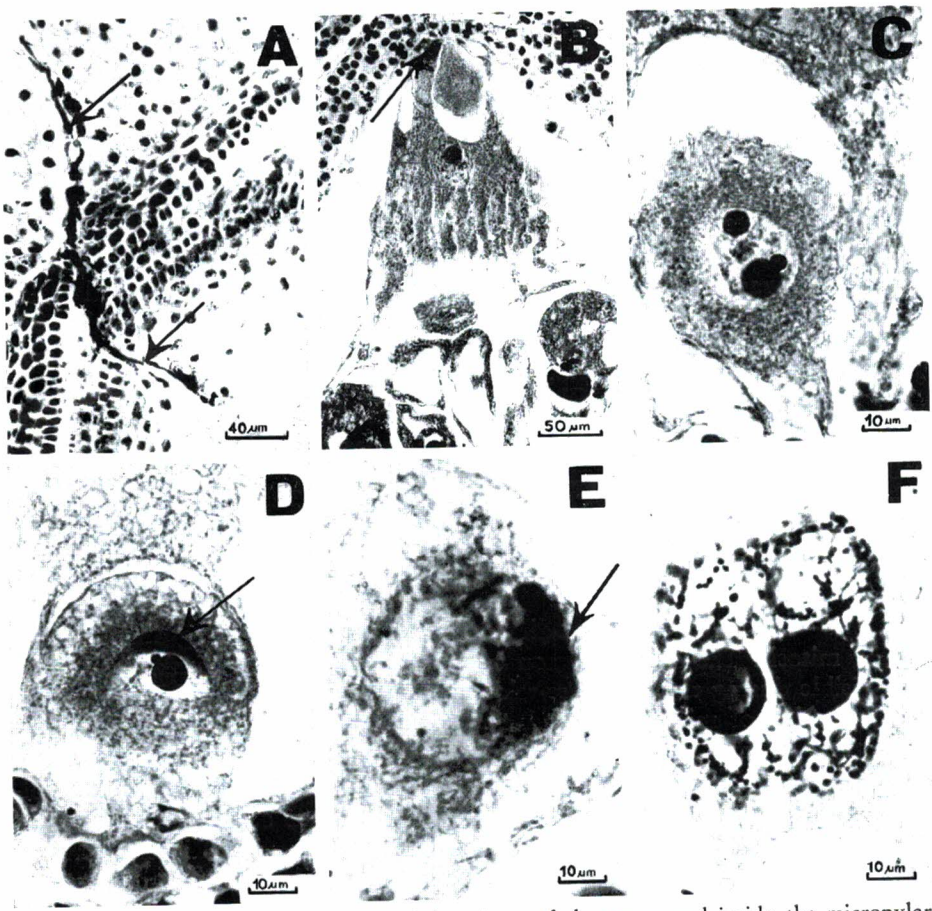

Fig. 2 A Pollen tube inside the transmitting tissue of the ovary and inside the micropylar end of the ovule (arrows) in the cross 'Bali' × *H. bulbosum*, 1 hour after pollination; B Residues of the pollen tube inside a synergide cell (arrow) in the cross 'Bali' × *H. bulbosum*; C Egg cell after fertilization in the cross 'Bali' × *H. bulbosum*, 3 d.a.p.; D Enlarged male gamete (arrow) after fusion with the egg cell nucleus in the cross 'Janus' × *H. bulbosum*, 6 d.a.p.; E Male gamete fused with polar nucleus in the cross 'Janus' × *H. bulbosum*, 3 d.a.p.; F Polar nuclei with small chromatin bodies 14 hours after pollinating 'Janus' with *H. bulbosum*

In enlarged caryopses 10—14 days after pollination, embryos usually reached stages shown in *Figure 1 K*. Only in 4 cases embryos with developed scutellum were found. During the next few days caryopses showed signs of abortion and therefore 12—14 days after pollination, the embryos were dissected and transferred to the medium. 46 embryos were isolated at various stages. Some embryos did not develop further and died after a few days of culture, some started to grow but produced only the root system. However, 20 embryos developed normally and from these 20 plants were obtained (*Fig. 1 M*). All 20 plants obtained were cytologically examined and chromosome counts made on root cells showed 21 chromosomes (*Fig. 1 L*).

Triticum aestivum **cvs. 'Bali' and 'Janus'** $\times$ ***Hordeum bulbosum***

As emphasized earlier, pollen tube germination, pollen tube growth and its penetration into embryo sacs were similar to those in crosses made with 'Chinese Spring'. The pollen tube also entered the embryo sac in the vicinity or through one of the synergids and usually was bursting there (*Figs. 2 A* and *B*). One male gamete entered the egg cell and the second migrated through the band of cytoplasm towards the polar nuclei. Later on, one male gamete was visible inside the egg cell and another one seemed to fuse with the polar nuclei (*Figs. 2 C* and *E*). It is not clear whether the male gamete which entered the egg cell fused with the nucleus. Probably, in some cases, this process had not occurred as the male gamete was still visible inside the nucleus even 6 days after pollination (*Fig. 2 D*). As distinct from the situation in the zygote, the male gamete which entered the polar nuclei fused with them and, therefore, a few hours after pollination the gamete was no more visible as a separate entity inside the polar nucleus. It is probable that both gametes fused and started to divide, but did not develop beyond the early stage of prophase. As a rule, 2—12 hours after pollination, additional nucleoli often appeared in the polar nuclei or a great number of small chromatin bodies situated on the chromosomes (*Fig. 2 F*). It was also quite usual that in embryo sacs analyzed a few hours after pollination the chromosomes of polar nuclei were very distinct and resembled structures similar to nuclei which were at the prophase stage. Beyond this stage, nuclei in the metaphase division were never found, nor any indication of the formation of endosperm nuclei.

Discussion

The results obtained in this study indicate that fertilization occurred in all 3 varieties of *Triticum aestivum* but only in 'Chinese Spring' the zygote was capable to divide. In 'Bali' and 'Janus' no signs of division induction of the egg cell or zygote were observed despite the fact that entrance of male gametes into both the egg cell as well as into polar nuclei was detectable. The analysis of the fertilization process in 'Chinese Spring', 'Bali' and 'Janus' shows to be essentially the same concerning the initial stages of the entrance of male gametes. In the case of 'Chinese Spring', after a complete fusion of the male gamete with the egg cell nucleus, the zygote fell into a period of dormancy and started divison only later. During the first few hours after fertilization it

was not possible to distinguish the male chromatin from the female one. This fact might indicate that fusion between two different gametes was complete and that from now onward the zygote went through a resting stage. The exact duration of this stage was not ascertained as the first few celled proembryos were only found 3 days after pollination. A similar process occurred with the second male gamete which fused with the polar nuclei but, unfortunately, also in this case the period of dormancy of the fused nuclei was not established. In some embryo sacs the endosperm did not develop and only small proembryos were present in the micropylar regions. It is probable that in these cases proembryos were not capable of dividing further and, therefore, degenerated later on.

In the case of 'Bali' and 'Janus' the male gametes reached the egg cell and the polar nuclei, but it is not clear whether a complete fusion took place. The uncertainty of this stems from the fact that no chromatin fusion between the male gamete and the egg nucleus was observed and that in some embryo sacs, even few days after pollination, the male gamete was visible inside the egg cell as a separate structure. Although a fusion between the chromatin of the male gamete and the polar nucleus took place, further development of the primary endosperm nucleus was never noticed.

Our investigations revealed that there exists an incompatibility system of *Hordeum bulbosum* with 'Bali' and 'Janus' and that this system works after the entrance of male gametes into the female cells. It is not clear what the nature of this system is. As postulated by CHAPMAN, RILEY and MILLER (1976) chromosomes 5 A and 5 B of 'Chinese Spring' may be responsible for the crossability of 'Chinese Spring' with *H. bulbosum.* Also according to the same authors chromosomes 5 A and 5 B are the location of the known genes for crossability with rye.

There are different modes of origin of parthenogenetic haploids and, as discussed by CHASE (1969), the maternal monoploid sporophytes in maize can arise from the precocious division of the unfertilized egg or another haploid cell of the embryo sac. The frequency with which this division can occur may be related to the stimulation by different agents, as shown in the literature dealing with the production of barley and wheat haploids by use of *H. bulbosum.* These haploids were obtained from hybrid zygotes only. The latter underwent divisions and during early stages of embryogenesis the male partner chromosomes were eliminated. There is no information available about the induction of mitosis of the egg nucleus prior to fertilization which is in agreement with our observation. Selective elimination of *H. bulbosum* chromosomes is quite a widespread phenomenon in *Hordeum* and the genetic control of chromosome elimination is already discussed in detail by several authors. In general, all the studies agree that selective elimination of *H. bulbosum* chromosomes occurred during haploid production. However, DAVIES (1958) who obtained a diploid *vulgare* progeny from the cross between tetraploid *H. bulbosum* and *H. vulgare,* suggested that the progeny resulted from male parthenogenesis. Also LANGE (1971) reported that the chromosomes of either *bulbosum* or *vulgare* may be eliminated.

It seems that in the cross 'Chinese Spring' × *H. bulbosum* a similar mechanism functions as in the case of *Hordeum vulgare,* but it is still uncertain what

factor(s) is (are) responsible for the inhibition of the zygote to divide in 'Bali' and 'Janus'. Further experiments are needed to determine whether in other varieties of *Triticum aestivum* the same or different processes occur as those in 'Bali' and 'Janus' after crossing with *H. bulbosum*. Only then would it be possible to determine whether the method of pollination of *Triticum aestivum* with *Hordeum bulbosum* will provide the plant breeder with desirable pure line varieties from sufficiently large numbers of haploids.

Summary

Fertilization was studied in the varieties of 'Chinese Spring', 'Bali', and 'Janus' of common wheat (*Triticum aestivum* L.) after pollination with *Hordeum bulbosum* (2n = 14). The pollen grains germinated almost immediately after attaching themselves to the stigmas and in about 30—40 minutes the pollen tubes reached the embryo sacs. Fertilization occurred in all the three varieties, but only in 'Chinese Spring' was the zygote capable to divide further. Only in this cross embryos developed concomitantly with a very poor endosperm. In some embryo sacs, the endosperm did not develop and proembryos degenerated early. During embryogenesis chromosome elimination was observed. Usually caryopses started to abort 12 days after pollination. Therefore, not fully formed embryos were dissected out and transferred to medium. Altogether, 46 embryos at various stages were isolated from which 20 fully developed haploid plants were obtained. When 'Bali' and 'Janus' were pollinated with *H. bulbosum* the male gametes entered into the egg cells and into polar nuclei, but neither the zygotes nor the primary endosperm nuclei have ever begun to develop.

Zusammenfassung

Cytoembryologische Untersuchungen des Befruchtungsvorganges und die Entwicklung haploider Embryonen bei *Triticum aestivum* L. (2n = 42) nach Kreuzung mit *Hordeum bulbosum* (2n = 14)

Bei den Weizensorten (*Triticum aestivum* L.) 'Chinese Spring', 'Bali' und 'Janus' wurde die Befruchtung nach Bestäubung mit *Hordeum bulbosum* (2n = 14) untersucht. Die Pollenkörner keimten, sobald sie auf die Narben kamen, und in etwa 30 bis 40 Minuten erreichten die Pollenschläuche die Embryosäcke. Bei allen drei Sorten fand eine Befruchtung statt, aber nur bei 'Chinese Spring' kam es zu weiteren Teilungen der Zygote. Nur bei dieser Kreuzung entwickelten sich neben einem sehr schwachen Endosperm Embryonen. In einigen Embryosäcken bildete sich kein Endosperm, und die Proembryonen degenerierten früh. Während der Embryogenese wurde Chromosomenelimination beobachtet. Normalerweise begannen die Karyopsen zwölf Tage nach der Bestäubung abzufallen. Deshalb wurden nicht völlig ausgebildete Embryonen herauspräpariert und auf Nährboden gesetzt. Insgesamt wurden 46 Embryonen verschiedener Stadien isoliert, aus denen 20 voll entwickelte haploide Pflanzen

entstanden. Wenn 'Bali' und 'Janus' mit *H. bulbosum* bestäubt wurden, gelangten die Gameten in die Eizellen und in die Polkerne, aber weder Zygoten noch Kerne des primären Endosperms begannen sich zu teilen.

This research was made possible by a fellowship awarded to the first author by the Max-Planck-Gesellschaft. Thanks are due to Miss B. MERKEL for technical assistance.

References

BARCLAY, I. R., 1975: High frequencies of haploid production in wheat (*Triticum aestivum*) by chromosome elimination. Nature (London) **256**, 410—411.

CHAPMAN, V., R. RILEY, and T. E. MILLER, 1976: The production of wheat haploids by the use of *Hordeum bulbosum*. Ann. Rep., Plant Breed. Inst., Cambridge, England, 99—101.

CHASE, S. S., 1969: Monoploids and monoploid-derivatives of maize (*Zea mays* L.). Bot. Rev. **35**, 117—167.

DAVIES, D. R., 1958: Male parthenogenesis in barley. Heredity **12**, 493—498.

— —, 1974: Chromosome elimination in inter-specific hybrids. Heredity **32** (2), 267—270.

D'SOUZA, L., 1972: Staining pollen tubes in the styles of cereals with cotton blue; fixation by ethanol-lactic acid for enhanced differentiation. Stain Techn. **47**, 107—108.

FEDAK, G., 1977: Haploids from barley × rye crosses. Canad. J. Genet. Cytol. **19**, 15—19.

GAMBORG, O. L., R. A. MILLER, and K. OJIMA, 1968: Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50**, 151—158.

KASHA, K. J., 1974: Haploids from somatic cells. In: K. J. KASHA (Ed.), Haploids in Higher Plants, 67—87. Univ. Guelph.

— —, and K. N. KAO, 1970: High frequency haploid production in barley (*Hordeum vulgare* L.). Nature (London) **225**, 874—876.

LANGE, W., 1969: Cytogenetical and embryological research on crosses between *Hordeum vulgare* L. and *H. bulbosum*. Versl. Landbouwk. Onderr. **719**, 162.

— —, 1971: Crosses between *Hordeum vulgare* L. and *H. bulbosum* L. II. Elimination of chromosomes in hybrid tissues. Euphytica (Wageningen) **20**, 181—194.

SUBRAHMANYAM, N. C., 1977: Haploidy from *Hordeum* interspecific crosses. Theor. Appl. Genet. **49**, 209—217.

— —, and K. J. KASHA, 1973: Selective chromosomal elimination during haploid formation in barley following interspecific hybridization. Chromosoma **42**, 111—125.

Authors' addresses: Prof. Dr. M. ZENKTELER, Institute of Biology, The University of A. Mickiewicz, Poznań (Poland); Prof. Dr. J. STRAUB, MPI für Züchtungsforschung, D-5000 Köln 30 (Germany F.R.).