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CLEARED-OVULE TECHNIQUE USED FOR RAPID ACCESS TO EARLY EMBRYO DEVELOPMENT IN *SECALE CEREALE* × *ZEA MAYS* CROSSES

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Nine rye genotypes and pollen mixture of three maize cultivars were evaluated for embryo formation in rye × maize crosses. Based on cleared ovule preparations, the development of embryo and endosperm were observed at 48, 72 and 96 h after pollination. Embryos were formed in eight cross combinations (3.3% to 23.3% depending on rye genotype), but in five combinations ovules had both embryos and endosperm nuclei. In a sample of 344 cleared ovules, 28 (8.1%) had both embryo and endosperm, 15 (4.4%) had only embryo, and 13 (3.8%) had only endosperm.

Key words: *Secale cereale*, *Zea mays*, embryo formation, endosperm, cleared-ovule technique, wide crosses.

INTRODUCTION

Interspecific and intergeneric crosses followed by rapid and complete elimination of the genome of the male parent have been an alternate method for inducing haploid zygotic embryos and subsequent plants. This technique was first introduced in cereals for the production of haploid barley plants with *Hordeum bulbosum* pollen (Kasha and Kao, 1970). Haploid plants obtained from crosses between wheat and maize were first reported by Laurie and Bennett (1988). Refinements of the technique (Suenaga and Nakijama, 1989; Laurie et al., 1990; Comeau et al., 1992) enabled haploid plants to be produced from many commercial wheat cultivars (Laurie and Reymondie, 1991; Riera-Lizarazu et al., 1992). Intergeneric triticale × maize crosses are an alternative way to produce haploid triticale (Wędzony et al., 1998).

Rye is known as a recalcitrant species in crosses with maize; only a few embryos were observed by Zenkteler and Nitsche (1984) and Laurie et al. (1990). Deimling et al. (1994) and Altenhofer et al. (1997) each obtained only two rye plants. There have been several reports on successful production of plantlets from anther culture of rye (Immonen, 1999; Immonen and Anttila, 1999, 2000). A problem in this technique is the formation of numerous of chlorophyll-deficient plantlets and very low regeneration efficiency.

This study examined the frequencies and rates of embryo and endosperm development, using cleared-ovule technique, in nine rye × maize combinations.

MATERIALS AND METHODS

In rye × maize crosses, nine rye genotypes (Kier, Strzekęcińskie, 5R, NR, S 888/99, S 726/98, S 727/98, S 828/98, S 830/98 from the Danko Plant Breeding Station in Choryń and the Plant Breeding Station in Nagradowice, Poland) were used as female plants. Rye plants were grown in potted soil in a greenhouse controlled at 25/14°C (day/night). Florets of *Secale cereale* were emasculated 1–2 days

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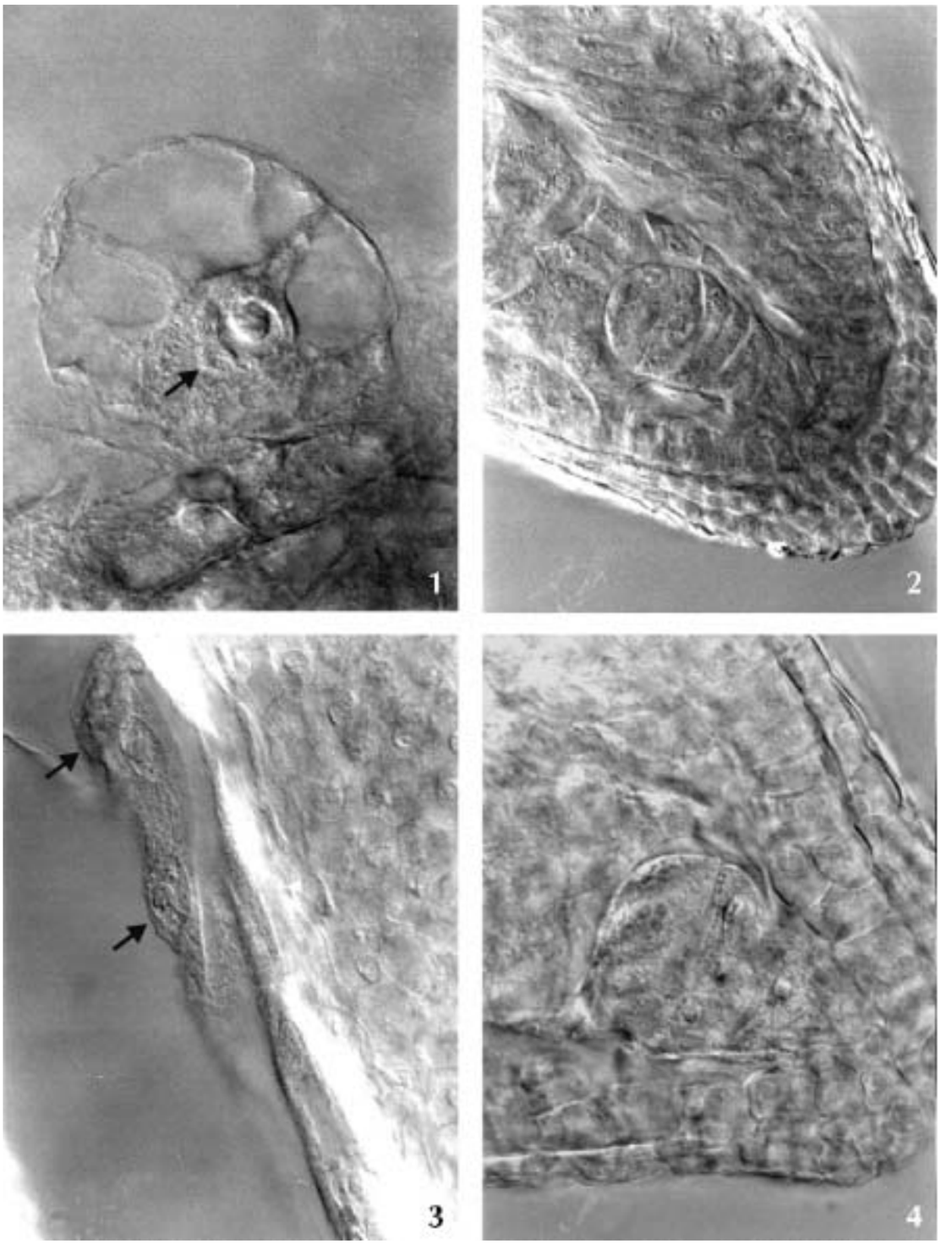

Figs. 1–3. *Secale cereale* cv. Strzekęcińskie × *Zea mays* (pollen mixture of Melba, Mieszko and Złota Karła cultivars). **Fig. 1.** Male gamete (arrow) in egg cell (24 h after pollination). × 210. **Fig. 2.** Several-celled embryo (48 h after pollination). × 70. **Fig. 3.** Endosperm nuclei (72 h after pollination). × 140. **Fig. 4.** *Secale cereale* cv. Kier × *Zea mays* – multicellular embryo (96 h after pollination). × 70.

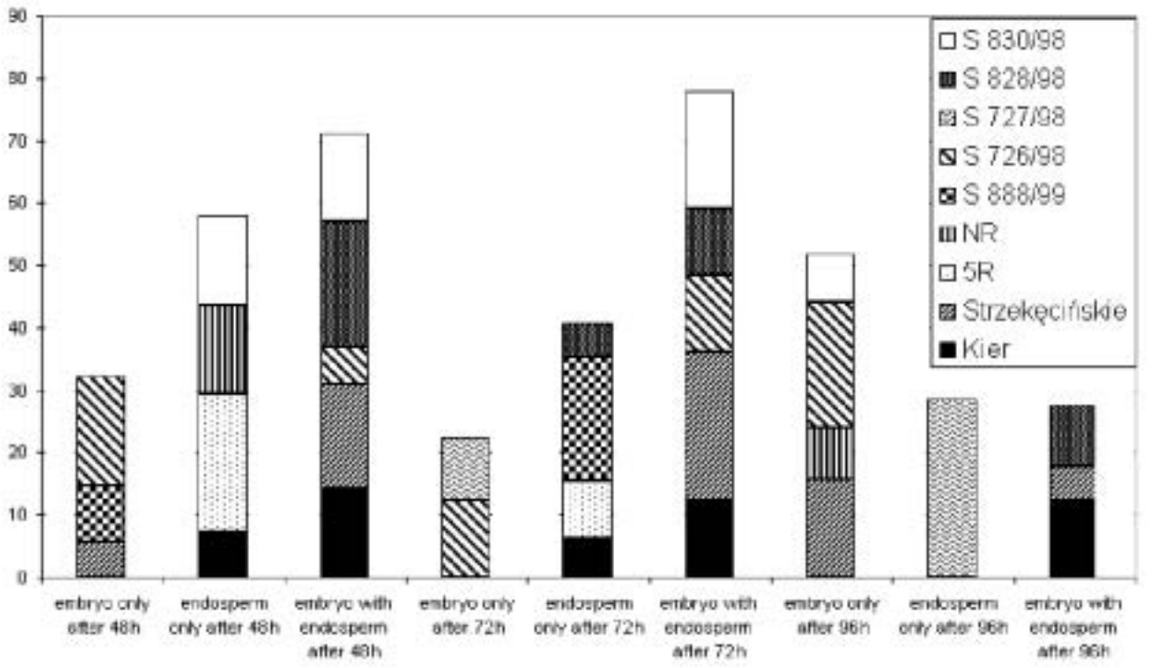

Fig. 5. Frequency (%) of embryo and endosperm development in *Secale cereale* × *Zea mays* crosses.

before anthesis and pollinated with freshly prepared pollen mixture from three maize cultivars (Melba, Mieszko, Złota Karła) at least twice on successive days. Maize plants were grown in soil in a greenhouse. Temperatures were ~35/10˚C (day/night) at anthesis of maize.

Pollen germination on the stigma was observed by fluorescence microscopy after staining with decolorized aniline blue (O'Donoughue and Bennett, 1994). For stimulation of embryo development, pollinated florets were dipped into aqueous solution of dicamba (75 mg/l). The pistils were fixed at 24 h intervals in FAA (40% formalin : glacial acetic acid: 70% ethanol, 5 : 5 : 90) for 24 h and stored in 70% ethanol. Then ovules were dissected from the pistils, dehydrated for 1 h in 70%, 80%, 90% ethanol (one change) and 100% ethanol (three changes), and cleared (for 1–2 h) in one change of ethanol : methyl salicylate (1 : 1), one change of ethanol : methyl salicylate (1 : 3) and two changes of 100% methyl salicylate (Young et al., 1979). Cleared ovules were stored in methyl salicylate in vials. The preparations were made according to Herr (1971). The slides were examined with a Nomarski interference contrast microscope (Nikon Diaphot-TMD). Ten days after pollination, swollen ovaries were aseptically dissected under a stereomicroscope for the presence or absence of an embryo for embryo culture.

RESULTS AND DISCUSSION

Pollen tube observations showed that the maize pollen mixture germinated on the rye stigmas in all cross combinations. Only some tubes grew through the transmitting tissue in the pistils and reached the ovules. There were several examples of a pollen tube entering a synergid, a male gamete in a fused polar nucleus, and the zygote and central cell in division. Figure 1 shows the stage before fusion of a sperm nucleus with the egg cell nucleus, 24 h after pollination. Embryo sacs with antipodal cells, polar nuclei, and an egg apparatus were found, similar to unfertilized female gametophytes. Few-celled or multicellular embryos and free-nuclear endosperm or partially cellular were observed in eight combinations at 48, 72 and 96 h after pollination (Figs. 2–4). Cellular endosperm was observed only in the micropylar region. The presence of endosperm nuclei suggested that the polar nuclei had fused with a maize sperm nucleus. However, fusion of the nuclei was

TABLE 1. Efficiency of embryo and endosperm formation in rye × maize crosses (48–96 h after pollination); % in paranthesis

Secale cereale	No. of ovules	Only embryo	Only endosperm	Embryo + endosperm	Total embryos
Kier	46	0	2 (4.3)	6 (13.0)	6 (13.0)
Strzekęcińskie	54	4 (7.4)	0	8 (14.8)	12 (22.2)
5R	28	0	3 (10.7)	0	0
NR	27	1 (3.7)	1 (3.7)	0	1 (3.7)
S 888/99	30	1 (3.3)	2 (6.7)	0	1 (3.3)
S 726/98	43	7 (16.3)	0	3 (7.0)	10 (23.3)
S 727/98	29	1 (3.4)	2 (6.9)	0	1 (3.4)
S 828/98	44	0	1 (2.3)	6 (13.6)	6 (13.6)
S 830/98	43	1 (2.3)	2 (4.7)	5 (11.6)	6 (13.9)
Total	344	15 (4.4)	13 (3.8)	28 (8.1)	43 (12.5)

not observed, and we have no proof for the autonomous origin of the endosperm.

Figure 5 illustrates the frequency and rate of embryo and endosperm development in nine crosses of rye and maize. Usually the ovules contained both an embryo and endosperm, but they sometimes contained embryos without endosperm or only endosperm. In wheat × maize crosses, Zhang et al. (1996) reported fertilized seeds containing an embryo or endosperm nuclei, but not both; Wędzony and Van Lammeren (1996) observed sometimes both embryo and endosperm, but usually embryos without endosperm or only endosperm.

In our experiments, embryo efficiency was markedly influenced by rye genotype and ranged from 3.3% to 23.3% in cross combinations. In a sample of 344 ovules cleared, 28 (8.1%) had both embryo and endosperm, 15 (4.4%) contained only embryo, and 13 (3.8%) only endosperm. The highest percentage of embryos was obtained when rye genotypes S726/98 (22.2%) and Strzekęcińskie (23.3%) were used (Tab. 1). Ten days after pollination, only degenerated embryos surrounded by liquid tissue replacing the endosperm were dissected from swollen ovaries. Zenkteler and Nitzsche (1984) observed embryos in embryo sacs of *Secale cereale* pollinated with *Zea mays*. Globular embryos were formed, but degenerated six to ten days after pollination. Endosperm development often appeared normal, sometimes without embryos. In all cases, however, endosperm development ceased within 10 days after pollination. Laurie et al. (1990) reported that fertilization occurred in 18.7% of florets when rye was pollinated with maize. Most fertilized florets had both embryo and endosperm (in 28 fertilized ovules, 14 had both embryo and endosperm; 10, only endosperm; and 4, only embryos). Ovules with both embryo and endosperm prevailed in our study also.

In wheat × maize crosses, embryos were observed, but their frequency depended on the parent genotype. In wheat ovules 48 h after pollination, Laurie et al. (1990) found that 23.3% had only an embryo, 2.3% had only endosperm, and 3.5% had both. In all of 21 wheat varieties pollinated with 'Seneca 60' maize, Laurie and Reymondie (1991) reported that 80.8% of florets in which fertilization had occurred contained only an embryo, 7.2% had only endosperm and 12.0% had embryo and endosperm. Suenaga et al. (1991) also obtained haploid wheat embryos from all crosses tested, using 47 wheat and 55 maize varieties and lines. Moreover, Wędzony et al. (1998) reported that the frequency of triticale embryos obtained from crosses with maize was equal to that of wheat, which usually responds well.

The cleared-ovule technique used in our experiments did not permit an exact analysis of maize chromosome elimination in the course of a few cell divisions in the hybrid embryo.

However, the technique enabled rapid assessment of fertilization frequency and early embryo development, which is very important for rye haploid production in rye × maize crosses. This technique was successfully applied in some wide crosses of cereals (Ślusarkiewicz-Jarzina et al., 2000).

In rye × maize crosses, embryos die already at early stages due to irregularities in endosperm development. Isolation of so young embryos for in vitro culture is very difficult or impossible. In further experiments, culture of proembryos inside the ovule 2–5 days after pollination may help to overcome this barrier, as Ślusarkiewicz-Jarzina et al. (1994) accomplished for plants obtained in intergeneric hybridization of the *Lolium-Festuca* complex.

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