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In vitro androgenesis of triticale in isolated microspore culture

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Abstract

Culture conditions for triticale (X *Triticosecale Wittmack*) androgenesis were studied using microspore culture. Sporophytic development of isolated triticale microspores in culture is described in five winter hexaploid triticale genotypes. Microspores were isolated using a microblendor, and embryogenesis was induced in modified 190-2 medium both in the presence and absence of growth regulators. The highest induction of microspore embryogenesis was obtained in a growth regulator-free medium. Adventitious embryogenesis was observed during *in vitro* development of triticale microspores. Albino and green plantlets were regenerated from embryo-like structures. More than 50% of regenerants were albino. In total, 126 green plantlets were produced, transplanted and established in soil. Cytological evidence revealed that 90% of the transplanted regenerants were haploid.

Abbreviations: ANOVA – analysis of variance; 2,4-D – 2,4-dichlorophenoxyacetic acid; ELS – embryo-like-structures; FDA – fluorescein diacetate; PAA – phenylacetic acid

Introduction

Triticale (X *Triticosecale* Wittmack) is a spontaneous synthetic amphiploid cereal which has been considerably improved through breeding (Rimpau, 1891; Immonen, 1996) and is currently grown in about 2 million hectares world-wide (Rajaram et al., 1993). Intensive research on triticale was started in the early 1950 (Kiss, 1966; Lelley, 1992). During the last decade, genetic studies (Lelley and Gimbel, 1989), somatic tissue culture (Stolarz and Lörz, 1986; Immonen, 1996), molecular genetics (Balatero et al., 1995; Wang et al., 1996) and transgenic studies (Zimny et al., 1995) have been reported.

Haploid tissue culture of several crops has been studied (Heberle-Bors, 1989) and used (Bajaj, 1990; Dunwell, 1996) and registered cultivars have been produced (Hu et al., 1986; De Buyser et al., 1987; Pauk et al., 1995; Khush and Virmani, 1996). Two *in vitro* cell and tissue culture methods are used to induce androgenesis from microspores: anther- and microspore culture. Since the induction of the first triticale anther culture-derived haploid plantlets by Wang et al. (1973), the method has been essentially refined (Bernard, 1980; Charmat and Bernard, 1984; Lukjanjuk and Ignatova, 1986; Martinez Garcia et al., 1992; Karsai et al., 1994). The use of potato extract in induction medium was suggested by Schumann and Hoffmann (1989). The benefits of anther pretreatment and use of conditioning anther culture media on triticale pollen development were reported by Keller (1991). Karsai et al. (1994) optimised the pH and maltose concentration in the induction medium. The beneficial effect of mannitol in the pretreatment solution and that of maltose in the induction medium was reported by Gland-Zwerger et al. (1994).

This report describes *in vitro* development of blendor-isolated triticale microspores leading to production of green and albino plantlets and the subsequent doubled haploid lines from microspore-

derived embryo-like structures. The effects of one growth regulator-free medium and two growth regulator-supplemented media on microspore embryogenesis were studied.

Materials and methods

Plant material

Seeds of winter triticale donor genotypes - a cv 'Presto' and four F1's (Tewo × Moniko, Presto × Novisadi, Presto × Moniko, Novisadi × Moniko) - were sown in the nursery in autumn. Each donor triticale genotype was a complete hexaploid (2n=6x=42, AABBRR). The donor cultivars used in the crosses were Polish 'Tewo', 'Moniko', 'Presto' and Yugoslavian 'Novisadi' and all are registered cultivars in Hungary. Donor tillers were cut between the 2nd and 3rd node with scissors when the primary anthers of the most mature florets contained mid- to late uninucleate microspores. Except for the flag leaf, the leaves were cut and the tillers were put into Erlenmeyer flasks containing fresh common tap water. Tillers were covered by a PVC bag to maintain high humidity. Microspore donor tillers were cold pre-treated under a dim fluorescent light at 4 °C for about two weeks.

The developmental stage of the micropores was determined microscopically after squashing the anthers in a drop of water. The number of isolated microspores was estimated with a haemocytometer. The different structures in microspore cultures were counted in representative fields using an inverted microscope.

Isolation of microspores

After cold treatment, about ten spikes containing the late uninucleate to early binucleate microspores were removed from the leaf sheath and surface-sterilised in 2% sodium hypochlorite for 20 min, and then rinsed three times with sterile water. The sterilised heads were cut into 1-cm sections and put into 100 ml Waring Micro Blendor container (Eberbach Corporation. Ann Arbor, Michigan, USA) and 60 ml of 0.3M autoclaved mannitol solution (5.47% mannitol in bidestillated water) was added. Microspores were isolated by blending twice for 5 sec at low speed, each time the quality of maceration was visually monitored through the plastic cap of the vessel. The crude microspore suspension was filtered through 160 and 80 μm sterile nylon sieves to remove raw spike debris. The filtrate was divided between four centrifuge tubes (each 10 ml volume) and centrifuged at 80 *g* for 5 min. The pellet was resuspended in 2 ml 0.3M mannitol and the microspore suspension was carefully layered over a 21% autoclaved maltose solution using a pipette. The solutions were centrifuged at 60 *g* for 10 minutes. The viable microspores were collected in a band at the maltose/mannitol gradient interphase using a Pasteur pipette. They were resuspended, washed in 0.3M mannitol (8 ml/tube) and centrifuged again at 60 *g* for 5 minutes.

Microspore culture

Following the final centrifuge, the pelleted microspores were resuspended in 1 ml of culture medium. The quantity of microspores was estimated using a Burker chamber. The viable microspores were identified by staining with fluorescein diacetate (Widholm, 1972). Subsequently, the culture density was adjusted to 0.9-1 $\times$ 10^5 microspores ml^{-1} by adding culture medium. Two ml of micropore suspension was put into a Greiner 35 mm plastic Petri dish. In the different experiments, four to seven replicates per genotype were made.

Parafilm-closed cultures were kept in darkness at 80% humidity at 28 °C. In the fifth week of subculture, the ELS (embryo-like-structures) were plated on Gelrite-solidified microspore culture medium and exposed to a 16-h photoperiod provided by cool white fluorescent tubes with 20 μmol m^{-2} s^{-1} irradiance, in a tissue culture chamber at 28 °C.

Regeneration and transfer of green plantlets

When the ELS reached the bipolar stage, the individual structures were transferred to 190-2 regeneration medium (Pauk et al.,1991) in glass culture tubes. The cultures were kept under a natural photoperiod. The well-tillered and rooted plantlets (about four weeks after subculture) were transplanted into non-sterilized 1:1 ratio peat/sandy soil. During the following two weeks, the plantlets were acclimatized in a Conviron growth cabinet at 80% humidity. The irradiance was 200 μmol m^{-2} s^{-1} with a 16-h photoperiod. Subsequently, the plants were grown under greenhouse conditions. Haploid plantlets were treated with colchicine before vernalization, as published for wheat anther culture-derived plantlets (Pauk et al., 1995). Vernalization was carried out in a cold chamber for six weeks at 2–4 °C under continuous fluorescent light at 40 μmol m^{-2} s^{-1} irradiance.

Culture media

For microspore culture induction media, the basic 190-2 medium (Zhuang and Jia, 1983) was supplemented with 3 mM L-glutamine and the following growth regulator combinations: 190-D/K = 1.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} Kinetin; 190-PAA = 10 mg l^{-1} PAA; 190-0 = without growth regulators.

Each culture medium included 175 mM maltose (Scott and Lyne, 1994) and the pH was adjusted to 5.8 with 1M KOH. Osmotic pressure was determined for each preparation using an osmometer. The media were filter sterilised and stored at room temperature, but medium older than four weeks was not used.

For subculture of induced ELS, Phytagel (2.5%) solidified microspore culture medium was used, and the 175 mM maltose content was reduced to 80 mM. For the regeneration of ELS collected from the plated cultures, 190-2 medium was used (Zhuang and Jia, 1983; Pauk et al., 1991).

Ploidy level determination

Chromosome numbers were determined from root meristem preparations. Donor plant root tips were pre-treated at 4 °C for 24 hours in a cool chamber. The pre-treated tips were collected and treated in a saturated oxiquinolin suspension for five hours and fixed in 3:1 ethanol and glacial acetic acid. Root tips were hydrolysed in 1N HCl at 60 °C for 10 minutes before staining in aceto carmine. Squash preparations were made in 45% acetic acid and chromosomes were counted in three wellspread cells from each root tip.

The length of stomatal guard cells was determined using an ocular micrometer from a 10-mm distal leaf segment taken from microspore-derived plants. Chlorophyll was extracted in 70% alcohol and the leaf segments were mounted in a drop of water on a glass slide with a coverslip. The length of stomatal guard cells was measured using a micrometer ocular. The length of stomatal guard cell of haploids was found to be 40–50% shorter than that of control hexaploid plants.

Analysis of data

The evaluation of data (Table 3 and 4) started with the descriptive statistical analysis (mean, standard deviation, coefficient of variation) of the three androgenetic parameters sorted by treatments (media). Standard deviation of data proved to be very high; therefore, logarithmic transformation for data of ELS was performed prior to further analysis. Data of albino and green plants given as percentage were transformed using arcsin transformation. After transformation, the data show approximately normal distribution making further analysis possible. ANOVA was performed for analysing the effects of the three media on the three androgenic traits. In the case of the regeneration of albino and green plants, oneway multiple comparison of the means of different media was based on the LSD. The mean values in ELS production were compared in case of similar deviations by a two sample t-test, and in the case of different deviations by the Welch-probe. ANOVA and other statistical tests (Fowler and Cohen 1990) were computed using appropriate programes from the MiniTab statistical package.

Results and discussion

Characteristic stages of triticale androgenesis in microspore culture

Viable, mid- and late-uninucleate microspores (Figure 1a) could be recovered from the blendor after the crude macerate had been sieved twice and centrifuged with a maltose/mannitol gradient (Figure 1b). The characteristic stages of triticale androgenesis were studied and the data were recorded (Table 1) for the F_1 cultures of Tewo-Moniko. FDA staining showed that 64% of the freshly isolated microspores were viable. After two days of culture, 25% of the viable micropores began cell division. The remaining microspores ruptured. Nine percent of the dividing cells produced multicellular colonies. These structures became visible under an inverted microscope within one week of isolation (Figure 1c). Thirty-five percent of the multicellular colonies were able to burst through the exine and develop into globular structures (Figure 1d). Within the next two-three weeks, they quickly developed into ELS, which were easily detectable on the surface of the culture medium (Figure 1e). During the fifth week of culture the structures were plated on solidified induction medium (Figure 1f) and after one-two weeks, the ELS were transfered to a regeneration medium, where they continued to grow. The well-developed ELS were separated and transferred into individual regeneration vials, where they germinated, producing green or albino plantlets. Nineteen percent of the proembryos developed into plantlets. After the eigth to nine week of culture, well tillered and rooted regenerants were transplanted into soil and

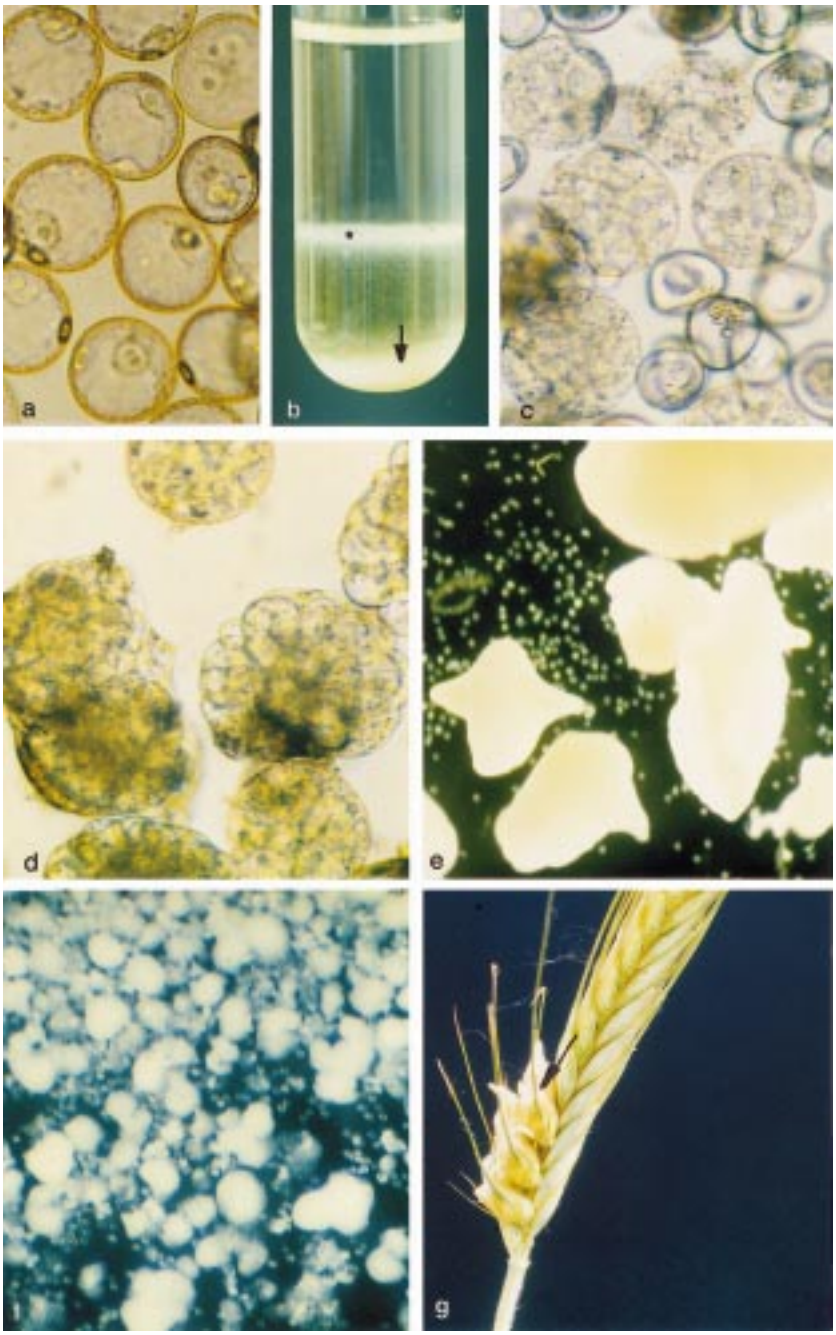

Figure 1. In vitro androgenesis of isolated triticale microspores: *(a)* freshly isolated late-stage uninucleated microspores in 0.3M mannitol, *(b)* separation of viable (star) and dead (arrow) microspores from blendor macerated crude spike extract on a maltose/mannitol cushion *(c)* dividing multicellular and non-responsive (dead) microspores one-week after isolation; *(d)* rapidly-growing microspore-derived aggregates two weeks after isolation, *(e)* ELS in the fourth week of culturing, non-divided microspores are visible in background; *(f)* ELS on a microspore culture medium before plating on the solidified medium, at five weeks after isolation; *(g)* microspore-derived colchicine-treated androgenetic triticale spike in isolation bag with fertile sector (arrow).

Table 1. Frequencies of development stages in isolated microspore culture of triticale, 'Tewo X Moniko' F_1 (average of 6 cultures)

Developmental stage	Microspore number/culture	Percentage*
Cultured microspores	9.5×10^4	
FDA stained microspores, after isolation	6.1×10^4	64
Viable microspores, 1 day after isolation	1.5×10^4	25
Multicellular colonies, 1 week after isolation	1.3×10^3	9
Globular structures, 4th week of culture	4.5×10^2	35
Plating efficiency, 6th week of culture	87	19

*Percentage in the previous developmental stage = $\frac{\text{No. of structures}}{\text{No. of previous developmental structures}} \times 100$

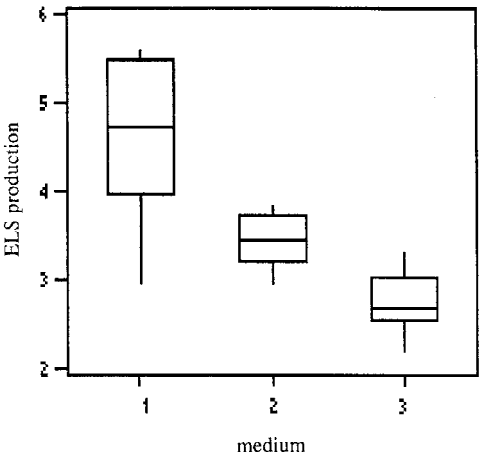

Figure 2. Boxplot from the effect of three different microspore culture media on ELS production. Logarithmic transformed data of Table 3 were used for the figure. Media: 1 – 190-0, 2 – 190-D/K, 3 – 190-PAA.

Table 2. Effect of genotype and culture medium on induction of ELS from triticale microspores

Genotype	Medium	Number of culture	ELS number/culture
Presto	190-0	3	20.0
	190-PAA	4	20.7
Tewo × Moniko F_1	190-0	6	11.3
	190-D/K	5	5.8
Presto × Novisadi F_1	190-0	1	3.0
	190-D/K	6	15.8
Presto × Moniko F_1	190-0	8	118.0
	190-PAA	1	7.0

grown further in the greenhouse. Well-tillered haploid regenerants were colchicine treated and after vernalization produced sterile, semi-fertile (Figure 1g) and fertile spikes.

Influence of growth regulator-free and growth regulator-supplemented media on triticale androgenesis

To collect data about the influence of genotype, medium on ELS production in micropore culture, an information experiment was made with the triticale cultivar 'Presto' and three different F_1 combinations (Table 2). Culture media with (190-PAA and 190-D/K) or without (190-0) exogenous growth regulators had a positive effect on ELS production for each genotype (Table 2). The highest number of ELS per culture (118) was recorded for 'Presto' x 'Moniko' in the 190-0 growth regulator-free induction medium. This culture medium induced 3 to 118 ELS per culture depending on genotype. The two different growth regulator-supplemented media (190-PAA, 190-D/K) also induced ELS at 5.8 to 20.7 per culture.

The significant positive effect of culture medium on ELS production was confirmed when green versus albino plant production was compared (Table 3). 190-0 medium had a significant influence on the ELS production, while the effect of this medium was not significant on regeneration of albino plantlets and there was only a weak effect on the green plantlet production. The addition of 2,4-D and Kinetin or PAA had no significant effect on any of the three parameters scored.

ANOVA (Table 4) showed that the effect of the media on the three traits was different and ELS variance due to differences of media was 16 times higher

Table 3. The values of mean (x), standard deviation (s), and coefficient of variation (CV%) of the investigated characteristics in three media based on logarithmic (ELS) and arcsin (albino and green plantlets) transformed data. Genotype: F_1 of 'Tewo' X 'Moniko' and seven replicates

In vitro traits	Medium	x*	s	CV%
ELS (number/culture)	190-0	4.64 a	0.95	20.5
	190-2.4D/K	3.44 b	0.33	9.6
	190-PAA	2.78 bc**	0.37	13.3
Albino plantlets (%)	190-0	28.00 a	9.12	32.6
	190-2.4D/K	17.56 a	10.61	60.4
	190-PAA	34.83 a	18.51	53.1
Green plantlets (%)	190-0	14.84 a	10.84	57.5
	190-2.4D/K	14.68 ab	8.85	60.3
	190-PAA	6.32 b	5.99	94.78

*Population means within a box having the same letters are not significant at p=0.05 level according to F protected LSD test.
**Significant at p=0.05 level according to two sample t-test.

than variance of error. This value decreased significantly in the case of albino and green plantlet regeneration. The medium effect was most significant (p=0.01) for ELS production, for green plantlet regeneration p=0.05, while the significance level for albino plantlet regeneration was only p=0.1. It means, the induction media had a strong effect on the number of ELS. In plantlet regeneration, the induction medium had less significant effect on green or albino plantlet regeneration.

The effect of different media on embryo production (ELS) was statistically confirmed. Values measured on 190-0 medium are higher than those on the other two media. 190-PAA medium gave the worst result, although it cannot be proved on 95% confidence level by ANOVA. Because standard deviation of data measured on 190-0 medium is many times higher than those measured on the two other media, means of the different media were compared using the Welch- test (Table 3). The results comfirmed that the 190-0 medium caused a higher level of embryogenesis and this effect is significantly higher either the 190-2,4D/K (p=0.05) and the 190-PAA (p=0.01) media. Two sample t-test showed the differences between 190-2,4D/K and 190-PAA media to be significant. The different medium influences on the ELS production was demonstrated in Figure 2. As there was no significant difference between the standard deviations of regenerated albino and green plants on the three different media, one way ANOVA was sufficient for the evaluation of the effect of media (Table 4). Different media caused no significant difference in the number of albino plants, but there was a significant difference at the 95% confidence level between 190-0 and 190-PAA media with respect to green plant production (Table 3).

Ploidy level of green plantlets

In total, 126 green regenerants were transferred to soil (Table 5). Regenerants were derived mainly from F_1 combinations and are therefore of potential breeding value and were maintained (Figure 1g). Before vernalization, the ploidy level of plants was determined by measuring the stomatal guard cell size and root tip cell chromosome count. The putative haploids had 40–50% shorter stomatal guard cells than the spontaneous or natural control hexaploids. The mixoploids had guard cells of an intermediate length or haploid and diploid length size were identified in different parts of the leaf. After this relatively quick test, 113 plants were determined to be haploid, 7 were mixoploid and 6 were spontaneous diploids. For 45 haploids, 7 mixoploids and 6 diploids a root tip chromosome count was done. A significant association was established between the results from assessing ploidy level using the two methods.

From the 113 colchicine-treated haploids, 89 gave at least one fertile seed. Test of the breeding value of the doubled haploid triticale lines is in progress.

Conclusions

The primary goal of the present study was to develop a simple isolation and culture method for triticale (X *Triticosecale* Wittmak) microspores for regeneration of doubled-haploid plants. It was demonstrated that regeneration of plantlets via direct isolation and culture of microspores is possible. The microspore culture protocol represents a novel way to induce triticale haploids, comparable with anther culture (Wang et al., 1973; Bernard, 1980; Lukjanjuk and Ignatova, 1986; Martinez Garcia et al, 1992; Karsai et al., 1994).

In triticale microspore culture the induction of androgenesis was successful when donor tillers were harvested at a later microspore developmental stage than for anther culture (data not shown). Harvest at the early- or mid-uninucleate stage of donor microspores for anther culture has been suggested to be optimal (Charmat and Bernard, 1984; Karsai et al., 1994). In

Table 4. Mean squares (MS) from the analysis of variance of microspore-derived ELS, regenerated albino and green plantlets of 'Tewo' × 'Moniko' F_1 triticale combination for microspore culture and regeneration of plantlets

Source of variation	df	ELS		Albino plant %		Green plant %	
		MS	F-test	MS	F-test	MS	F-test
Medium	2	6.179	16.17***	530	2.95*	284.7	3.69**
Error	18	0.382		179		77.2	

Significant at the $^{*}p$=0.1, $^{**}p$=0.05, $^{***}p$=0.01 level

Table 5. Genotype and ploidy level distribution of triticale microspore culture regenerants. Number of root tips assessed in parentheses

Genotype	Transplanted plantlets	Ploidy level		
		n (3x)	mixoploid	2n (6x)
Presto	18	15 (10)	1 (1)	2 (2)
Tewo × Moniko F1	12	11	1 (1)	–
Presto × Novisadi F1	6	6	–	–
Presto × Moniko F1	19	19 (7)	–	–
Novisadi × Moniko F1	71	62 (28)	5 (5)	4 (4)
Total	126	113 (45)	7 (7)	6 (6)

our study, the late-uninucleate and early- binucleate stage was found to be the best for microspore culture, as in other cereal microspore culture reports (Hoekstra et al., 1992; Mordhorst and Lörz, 1993; Puolimatka et al., 1996). The blendor isolation protocol provided a sufficient number of micropores to avoid the necessity for anther isolation. Microblending isolation was earlier established for barley (Olsen, 1991; Scott and Lyne, 1994; Mordhorst and Lörz, 1993) and wheat (Ziauddin et al., 1992; Puolimatka et al., 1996); this study confirms its potential.

The separation of viable from dead cells by density gradient centrifugation was first suggested for protoplast preparations by Harms and Potrykus (1978) and the idea has since been successfully adapted to barley (Olsen, 1991; Mordhorst and Lorz, 1993) and wheat (Puolimatka et al., 1996).

The viable cytoplasmically dense microspores were successfully cultured in three different induction media, with a medium without growth regulators producing the best result. In cereals, there are two reports about growth regulator-free induction of androgenesis. In oats, high frequencies of anther culture derived embryos can be obtained in a growth regulator-free medium (Rines et al., 1992; Kiviharju et al., 1996) and similar results have been published for rapeseed (Telmer et al., 1995) and tobacco (Heberle-Bors, 1989).

Every genotype tested responded in the regeneration experiment. Some genotypes eg. 'Presto', 'Moniko', 'Tewo' were tested in a previous anther culture experiment (Karsai et al., 1994) and were found to be responsive. These genotypes maintained their good tissue culture response in crossing combinations and under microspore culture conditions indicating the genetic determination of this parameter in triticale (Bernard, 1980; Charmat and Bernard, 1984; Balatero et al., 1995).

Adventitious embryogenesis was observed in isolated microspore culture of triticale. This phenomenon also occurs in microspore culture of other cereals (Hoekstra et al., 1992; Ziauddin et al., 1992; Jähne et al., 1994). From the ELS, 126 green plants were regenerated. Albinism, which is a typical problem in triticale anther culture (Karsai et al., 1994), also remained a problem in microspore culture - more than half of the regenerants were albino.

The stomatal guard cell length and cytological examination of 126 microspore-derived plants indicated that mainly haploid (90%) plants were produced from microspore culture. In the case of 58 regenerants, the stomatal guard cell length result was confirmed by root tip cytology. This simple method is practical and the

results obtained here support those for barley (Borrino and Powell, 1988). The regenerants produced in these experiments will be used in a breeding program.

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