AGRICULTURAL AND FOOD SCIENCE

Vol. 13 (2004): 363–377.

Factors influencing somatic embryogenesis and regeneration ability in somatic tissue culture of spring and winter rye

Rui Ma and Seppo Pulli

Laboratory of Plant Physiology and Molecular Biology, Department of Biology, FI-20014 University of Turku, Finland, e-mail: seppo.pulli@koti.luukku.com

Rye is an important crop in Northern and Eastern Europe. However, the application of various biotechnologies in rye breeding has been limited duo to its recalcitrant in tissue culture. In order to improve somatic tissue efficiency, key factors affecting somatic embryogenesis and reproducible green plant regeneration of rye (*Secale cereale* L.) were evaluated and optimised. In this study, a total 27 rye genotypes including 10 spring and 17 winter genotypes were involved in the investigation. Genotype, culture medium, sugar, gel agent and auxin influenced somatic embryogenesis of immature embryo significantly. One-two weeks cold pretreatment of young embryo enhanced somatic embryogenesis and green plant regeneration. In culture of immature embryos, inflorescences and leaf segments of the seedlings, explants significantly influenced the culture efficiency. Highest embryogenic callus yield resulted from rye immature embryo as explant compared to young inflorescence and leaf segment of seedling. Developmental stage of embryo played an important role in somatic embryogenesis. Late spherical coleoptile stage (embryo size 0.5–1mm in length) was optimal developmental stage of immature embryo for culture. Morphogenetic potential of embryogenic callus decreased with an increasing number of subcultures, and this ability could be maintained *in vitro* for a maximum of 8 months of culturing.

Key words: rye, somatic embryogenesis, embryo, inflorescence, leaf segment

Introduction

Various biotechnological applications in plant breeding, such as gene transformation and *in vitro* selection, rely on the availability of efficient plant tissue culture systems. Regeneration of plants from in vitro-cultured cells is an important advance in the genetic manipulation of plants (Lörz et al. 1988). Plant tissue and cell culture techniques

Manuscript received January 2004

have long been recognized as valuable tools in crop improvement programs, particularly in crop breeding. Efficient plant tissue culture systems have been established and advances made in the genetic transformation of staple cereals, including wheat (Vasil et al. 1993, Zhang et al. 2000), rice (Dong et al. 2001, Pragya et al. 2002) and barley (Wan and Lemaux 1994, Trifonova 2001).

Rye (*Secale cereale* L. 2n = 2x = 14) is an important cereal crop in Europe, and its adaptability to adverse conditions, such as low temperatures and diseases, among others, is also of major interest to plant breeders. However, rye has proved to be among the most recalcitrant graminaceous species in plant tissue culture and genetic transformation. So far, transgenic rye plants have been reported (Castillo et al. 1994, Popelka and Altpeter 2003a, b, Altpeter et al. 2004), and the target materials were limited in specific inbred line. The lack of an efficient *in vitro* culture system is still a limitation for genetic transformation and manipulation of this species. Early investigations of rye somatic embryogenesis and plant regeneration from various explant sources of immature embryo (Rybczynski 1979, Zimny and Lörz 1989, Rakoczy-Trojanowska and Malepszy 1995, Popelka and Altpeter 2001), young inflorescence (Krumbiegel-Schroeren et al. 1984, Linacero and Vazquez 1990, Rakoczy-Trojanowska and Malepszy 1993), young leaf segment (Linacero and Vazquez 1986) and root organ cultures (Whitney 1996) have been described in several reports. However, the low embryogenesis and green plant regeneration are problematical. In particular, the effects of some important factors, such as different explants, cold pretreatment, genotype, medium, auxins and sugars, influencing somatic embryogenesis remain obscure.

In this study, the important physiological and physical factors influencing embryogenic callus induction and reproducible green plant regeneration were evaluated and optimised to improve efficiency of plant tissue culture systems of rye in practical rye breeding work for genetic transformation, manipulation and in vitro selection. Embryogenic calli from somatic tissue culture were also suitable sources for cell suspension culture initiation and protoplasts culture.

Material and methods

Plant materials

A total of 27 rye cultivars including 10 spring and 17 winter genotypes served as donor plants in this experiment (Table 1). Plant materials, which were used for immature embryo culture and inflorescence culture, were grown in a greenhouse (18/16ºC, day/night temperature, 16-h photoperiod at about 200 µmol $m^{-2}s^{-1}$). Seedlings of winter genotypes were vernalized in a cold room (4ºC, 12-h photoperiod at 70 µmol $m^{-2}s^{-1}$ light intensity) for 10–12 weeks then transferred to the greenhouse. For leaf segment culture, sterilized seed (surface sterilized by 1.6% sodium hypochlorite with 0.5 Tween 20 for 15 min then rinsed several times with sterile water) of rye cultivars was germinated in Magenta boxes with sterilized filter paper at

Table 1. Source of rye materials in experiment.

Eco-type	Genotype	Source
Spring	Auvinen	Finland
	ME-80083	Finland
	OD	Sweden
	Vågones vårrug	Norway
	KVL-7002	Denmark
	Rogo	Germany
	Florida dwarf	USA
	Florida 401	USA
	Kalhek K131	Afghanistan
	Gansu	China
Winter	Anna	Finland
	Riihi	Finland
	Jussi	Finland
	Akusti	Finland
	Voima	Finland
	Elvi	Estonia
	Sangaste	Estonia
	Vambo	Estonia
	Vågones	Norway
	Amilo	Poland
	Danko	Poland
	Zulpan	Russia
	EM-1	Russia
	Bylina	Ukraina
	Bonel	USA
	Wheeler	USA
	Lanzhou	China

Vol. 13 (2004): 363–377.

24ºC in darkness, and then grown at 24ºC/18ºC (day/night) under a light regime of 16-h photoperiod at 80 μmol $m^{-2}s^{-1}$.

Embryogenic callus induction

Spikes were harvested 10–20 days after isolated pollination (spikes isolated with cellophane bags to prevent cross pollination from other genotypes), and were stored at 4ºC in darkness with stalks in water. Immature caryopses were surface-sterilized by 1.6% sodium hypochlorite with 0.5% Tween 20 for 15 min then rinsed several times with sterile water. Immature embryos were placed on an induction medium with scutellum up. Tillers containing young inflorescence 0.5–2 cm in length were collected, and surface-sterilized in 70% ethanol for 30 seconds. Inflorescences were cut into 2-mm-long segments and cultured on an induction medium. For leaf segment culture, basal part of shoot (approx. 30 mm, 2–3 weeks old) grown in Magenta boxes were cut into 3-mm-long sections and cultured on induction medium. Approximately 10 immature embryos, inflorescence segments or leaf sections were cultured in a Petri dish (diameter 95 mm) containing 30 ml solid induction medium.

Media MS (Murashige and Skoog 1962), CC (Potrykus et al. 1979), AA (Müller and Grafe 1978, omitted kinetin and GA3), mMS (modified MS medium consisting of MS basic salts and vitamins, 2 mg l^{-1} glycine, 146 mg l^{-1} glutamine, 200 mg l^{-1} casein hydrolysate, Li et al. 1992) and S_1 (Yin et al. 1993) consisting of macronutrients of AA medium, micronutrients and vitamins of B5 medium (Gamborg et al. 1968), 500 mg l^{-1} Proline, 877 mg l^{-1} glutamine, 266 mg l^{-1} aspartic acid, 288 mg l^{-1} arginine, 75 mg l^{-1} glycine, 20 mg l^{-1} coconut milk were used as induction media. pH of the media was adjusted to 5.8 before autoclaving. Coconut milk was added after autoclaving by filter sterilization. Cultures were incubated at 27ºC in darkness. The effects of different auxins (2 mg l^{-1} 2,4-D, Sigma, 4 mg l^{-1} Dicamba, Sigma, 2 mg l^{-1} NAA, Sigma, the concentrations used were optimal concentrations in our pre-tests), sugars (3% of sucrose, maltose and glucose) and gel agents (0.3% Phytagel, Sigma and 0.7% Agar, Difco) in AA medium on embryogenesis of immature embryos were tested. Totally 9 050 embryos were used in these three tests.

In cold treatment test, spikes with immature embryos were cold treated at 4ºC in darkness with stalks in water for different durations (0–5 weeks). A total of 3 600 immature embryos were used in the cold treatment experiment. Totally 1 808 immature embryos were used for test of genotypic effects. The culture abilities of different explants (immature embryos, inflorescence segments or leaf sections of seedling) were tested and 850 immature embryos, 850 immature inflorescence segments and 850 leaf sections were cultured. AA medium was used as embryogenic induction medium in these experiments.

Effects of developmental stages of immature embryos on embryogenesis were studied. Immature embryos in different developmental stages (1–6 scale of developmental stages of Zimny and Lörz 1989, embryo size from < 0.5 mm, 0.5–1 mm, 1–2 mm, 2–3 mm) were cultured on AA medium in this experiment. Totally 3200 embryos were used in this experiment.

Green plant regeneration

After 4–5 weeks incubation, frequencies of explants forming embryogenic calli were scored. To test regeneration capability, embyogenic calli were transferred onto 190-2 (Wang and Hu 1984) regeneration medium supplemented with 3% (w/v) sucrose, 3 mg l^{-1} BA, solidified with 0.45% Phytagel, and cultured at 21ºC and 16-h photoperiod (40 μmol $m^{-2}s^{-1}$). Partial embryogenic calli were maintained by monthly subculture on solid AA medium with 2 mg l^{-1} 2,4-D, and the morphogenetic competencies examined at monthly intervals.

Data collection and statistical analysis

Frequencies of explants forming embryogenic calli (embryogenic calli / 100 explants) and frequencies of regenerated calli producing green plants (green

plants / 100 calli) were recorded. In the experiments, the completely randomized designs (CRD) were used. Each Petri dish was considered an experimental unit and each treatment contained five replicates. Response variables were callus induction and green plant regeneration. Data analyses with more than two treatment levels were carried out by the ANOVA procedure. Multi-range comparisons were performed by LSD test.

Results and discussion

Medium effects on somatic embryogenesis

In the five media tested, AA medium produced a significantly ($P < 0.05$) higher embryogenic efficiency than CC and mMS media, and CC and mMS media, in turn, gave a significantly ($P < 0.05$) higher embryogenic efficiency than S_1 and MS media. No interactions of genotype × medium on somatic embryogenesis were detected ($P > 0.05$) (Table 2).

Culture medium is another important factor influencing somatic embryogenesis. In previous work on the somatic tissue culture of cereal crops, different basal culture media have been employed for embryogenic callus induction. MS or modified MS medium are most commonly used as induction media for somatic embryogenesis of wheat, rice and barley. CC medium has been recommended for embryo culture of rye (Zimny and Lörz 1989) and barley (Lührs and Lörz 1987). AA is an amino acid based medium preferred by Immonen (1996) for embryo culture of triticale. In this study, AA

Table 2. Effects of ecotypes, genotypes and media on somatic embryogenesis (embryogenic calli per 100 embryos) in immature embryo culture of rye (3% sucrose and 0.3% Phytagel were used). Each treatment contained five replicates.

Eco-type	Genotype	Medium				
		AA	CC	mMS	S1	MS
Spring	ME80083	72.35	67.50	56.58	35.00	37.50
	Florida dwarf	70.99	61.29	64.45	45.83	60.71
	Vågones vårrug	65.53	64.62	61.11	52.34	56.67
	Auvinen	63.88	57.04	53.13	44.36	39.50
	OD	61.54	45.45	51.25	38.75	35.38
	Gansu	61.30	32.23	44.30	39.38	30.83
	Rogo	63.33	62.50	55.92	48.33	41.89
	Average	65.56A	55.80B	55.25B	43.43C	43.21C
Winter	Zulpan	68.14	63.04	52.94	50.00	46.67
	Amilo	69.91	50.00	58.82	46.15	46.32
	Jussi	63.29	56.98	53.54	43.01	37.58
	Anna	66.59	62.71	63.92	54.00	49.00
	Riihi	57.07	51.36	48.80	31.25	31.54
	Vågones	60.61	54.33	56.25	38.55	35.05
	Wheeler	58.42	52.56	50.46	37.89	43.53
	Voima	54.38	49.52	48.95	38.60	37.43
	Average	55.38A	48.94B	48.19B	37.72C	36.35C
	Total average	63.82A	55.41B	54.69B	42.89C	41.97C

$LSD_{0.05}$ = 5.19 between media within spring genotypes
$LSD_{0.05}$ = 5.06 between media within winter genotypes
$LSD_{0.05}$ = 3.62 between media
AA medium (amino acid based medium, Müller and Grafe 1978)
CC medium (Potrykus et al. 1979)
mMS medium (modified MS medium, Li et al. 1992)
MS medium (Murashige and Skoog 1962)
S1 medium (Yin et al. 1993)

Vol. 13 (2004): 363–377.

medium produced the highest number of embryogenic calli, suggesting AA medium to be the induction medium of choice in practical plant tissue culture for somatic embryogenesis of rye. The rest experiments of this study were performed with AA medium (Tables 3, 4 5, 7, 8, 9, 10).

Influence of auxins on somatic embryogenesis

Different auxins 2,4-D, Dicamba and NAA were tested in this experiment. Significantly highest embryogenesis was from Dicamba comparing 2,4-D and NAA. No statistically significant interactions between genotype and auxin on embryogenesis were found. The highest embryogenesis frequency was from 'Florida dwarf' by Dicamba, the lowest was from 'Auvinen' with NAA (Table 3).

Efficiency of auxins on somatic embryogenesis in cereals has been assessed in previous works (Lührs and Lörz 1987, Popelka and Altpeter 2001). The type and concentration of auxins in the induction medium is important for obtaining a high efficiency of somatic embryogenesis (Zimny and Lörz 1989). Dicamba has proved to be more effective on somatic embryogenesis than 2,4-D in embryo culture of barley (Lührs and Lörz 1987, triticale (Immonen 1996) and rye (Zimny and Lörz 1989). In our study, Dicamba gave the best somatic embryogenesis compared to 2,4-D, while NAA was in accordance with the observations of Zimny and Lörz (1989).

Sugar and gelling agent in culture medium

In this study (Table 4) somatic embryogenesis was significantly ($P < 0.001$) influenced by sugars used in the induction medium. Significantly highest embryogenesis was produced by sucrose than by

Table 3. Effects of ecotypes, genotypes and different auxins on somatic embryogenesis (embryogenic calli per 100 embryos) in rye embryo culture. AA medium with 3% sucrose and 0.3% Phytagel was used for induction medium. Each treatment contained five replicates.

Eco-type	Genotype	Growth regulator		
		2,4-D (2 mg l^{-1})	Dicamba (4 mg l^{-1})	NAA (2 mg l^{-1})
Spring	Florida dwarf	66.74	79.17	51.79
	Vågones vårrug	58.66	67.17	52.35
	Auvinen	52.32	67.26	45.19
	Rogo	52.17	56.23	47.09
	Average	57.47B	67.46A	49.11C
Winter	Zulpan	66.46	71.25	58.06
	Amilo	62.85	66.15	50.00
	Jussi	61.99	70.49	52.38
	Anna	63.70	72.28	57.23
	Riihi	48.40	66.36	45.91
	Vågones	54.33	61.46	48.39
	Wheeler	56.48	60.39	51.94
	Voima	52.18	56.52	47.22
	Average	58.30B	65.61A	51.39C
	Total average	58.02B	66.23A	50.63C

$LSD_{0.05}$ = 7.78 between auxins within spring genotypes
$LSD_{0.05}$ = 5.45 between auxins within winter genotypes
$LSD_{0.05}$ = 4.42 between auxins
2,4-D: 2,4-dichlorophenoxyacetic acid
Dicamba: 3,6-dichloro-2-metoxy benzoic acid
NAA: naphthaleneacetic acid

maltose and glucose. No significant genotype and sucrose interactions were detected. Somatic embroygenesis was significantly improved by using the gel agent Phytagel (0.3%) ($P < 0.001$) compared to agar (0.7%) (Table 4).

Sugar is an essential requirement for all culture media, providing carbon, energy and osmotic regulation. Sugar influences callus induction and green plant regeneration. In cereal crop somatic tissue culture, sucrose is most frequently used in culture media for somatic embryogenesis. Maltose has been shown to be the most effective sugar for increasing androgenesis and green plant regeneration in rye (Flehinghaus et al. 1991), wheat (Moieni 1997) and barley (Kuhlmann and Foroughi-Wehr 1989, However, our results revealed maltose (3%) to be an inferior carbohydrate source in comparison with sucrose (3%) for somatic embryogenesis of rye.

Influences of gelling agent in plant tissue culture have been reported in previous studies (Kohlenbach and Wernicke 1978, Lührs and Lörz 1987, Flehinghaus et al. 1991). Agar was widely used for culture medium solidification. However, since Kohlenbach and Wernicke (1978) found the inhibitory effects of agar, alternative gelling agents, such as agarose and gelrite, have been employed to replace agar. Agarose was superior to agar for increasing the efficiencies of somatic embryogenesis in rye (Zimny and Lörz 1989) and barley (Lührs and Lörz 1987) embryo culture. Gelrite was better than agrose for embryogenic induction and regeneration rates in rye anther culture (Flehinghaus et al. 1991) and embryo culture (Popelka and

Table 4. Effects of ecotypes, genotypes, sugars and gel agents on somatic embryogenesis (embryogenic calli per 100 embryos) in rye embryo culture. AA medium with 4 mg l^{-1} Dicamba and 0.3% Phytagel was used for induction medium. Each treatment contained five replicates.

Eco-type	Genotype	Sugar			Gel agent	
		Sucrose (3%)	Maltose (3%)	Glucose (3%)	Phytagel	Agar
Spring	ME80083	64.06	57.83	53.95	63.85	47.50
	Florida dwarf	68.42	58.33	62.26	64.59	59.85
	Vågones vårrug	61.74	58.97	56.13	61.00	57.42
	Auvinen	56.48	48.74	41.21	61.38	55.21
	OD	57.86	48.33	45.94	58.57	51.25
	Gansu	61.95	52.70	48.11	58.44	51.85
	Average	61.75A	54.15B	51.27B	61.31a	53.85b
Winter	Zulpan	73.45	61.65	59.93	70.86	64.44
	Amilo	65.87	60.7	49.49	67.39	52.63
	Jussi	64.56	56.25	48.14	62.53	54.92
	Anna	56.95	47.67	44.33	57.56	53.91
	Riihi	54.93	49.24	43.78	54.14	39.72
	Vagones	53.89	47.31	50.00	56.25	38.46
	Wheeler	58.92	53.71	50.60	57.73	45.87
	Voima	52.47	48.43	46.54	51.80	47.74
	Average	60.13A	53.12B	49.10B	59.78a	49.71b
	Total average	60.83A	53.56B	50.03B	60.44a	51.48b

$LSD_{0.05}$ = 5.75 between sugars within spring genotypes
$LSD_{0.05}$ = 5.37 between sugars within winter genotypes
$LSD_{0.05}$ = 3.85 between sugars

$LSD_{0.05}$ = 5.92 between gel agents within spring genotypes
$LSD_{0.05}$ = 5.18 between gel agents within winter genotypes
$LSD_{0.05}$ = 3.87 between gel agents

Vol. 13 (2004): 363–377.

Altpeter 2001). Our results confirmed the superiority of Phytagel (Gelrite) over agar for somatic embryogenesis in the embryo culture of rye.

Cold pre-treatment on somatic embryogenesis and plant regeneration

Cold pre-treatment of immature embryo at 4ºC in darkness for 1–2 weeks significantly improved somatic embryogenesis (Table 5) and plant regeneration (Table 6). Duration of cold pre-treatment longer than three weeks reduced the culture efficiencies. No interactions of genotype × cold treatment on embryogenesis and plant regeneration were found.

In cereals cold pre-treatment of anthers is among the key factors influencing androgenesis and commonly employed to enhance the frequency of embryogenesis and green plant regeneration (Thomas et al. 1975, Guo and Pulli 2000). Cold pre-treatment of immature embryo was found to be favourable to somatic embryogenesis and plant regeneration in wheat (Maes et al. 1996) and triticale (Immonen 1996). A three-week cold pre-treatment of immature embryo at 4ºC before culture for wheat somatic embryogenesis and plant regeneration, and a two-week cold pre-treatment of immature embryos at 8ºC in culture for triticale plant regeneration were optimal. According to the results of our studies, one to two weeks of cold pre-treatment was beneficial for both the embryogenesis and plant regeneration of rye.

Differences of somatic embryogenesis between plant organs of rye

Somatic embryogenesis of different plant organs (embryo, inflorescence and leaf segment) of rye was tested (Table 7). Culture efficiencies (embryogenic calli per explant) for both spring and winter

Table 5. Effects of ecotypes, genotypes and cold pre-treatment on somatic embryogenesis (embryogenic calli per 100 embryos) in immature embryo culture of rye. AA medium with 3% sucrose, 4 mg l^{-1} Dicamba and 0.3% Phytagel was used for induction medium. Each treatment contained five replicates.

Eco-type	Genotype	Cold pre-treatment (weeks)					
		0	1	2	3	4	5
Spring	Florida dwarf	63.69	73.81	71.43	67.78	62.00	53.47
	Vagones vårrug	59.91	65.71	63.33	51.22	48.35	36.92
	Auvinen	60.44	63.78	60.03	43.76	39.55	34.87
	Gansu	64.29	65.69	66.67	52.75	50.77	35.70
	Average	62.08A	67.25A	65.37A	53.88B	50.17B	40.24C
Winter	Zulpan	64.33	73.84	72.22	63.64	62.71	56.82
	Amilo	64.86	68.09	69.35	58.31	55.20	52.27
	Jussi	53.51	74.04	68.02	48.74	38.41	41.23
	Anna	56.57	69.01	63.70	54.59	53.60	49.60
	Riihi	53.03	65.04	68.45	51.73	37.80	25.00
	Vågones	54.81	58.26	54.45	50.00	49.66	48.21
	Wheeler	57.58	60.93	60.66	56.88	53.79	48.94
	Voima	45.85	51.66	58.33	48.75	48.48	46.03
	Average	56.32B	65.11A	64.40A	54.08BC	49.96CD	46.01D
	Total average	58.24B	65.82A	64.72A	54.01BC	50.03C	44.09D

LSD0.05 = 5.69 between cold treatments within spring genotypes
LSD0.05 = 5.38 between cold treatments within winter genotypes
LSD0.05 = 4.25 between cold treatments

genotypes were considerably higher by embryo culture than by inflorescence culture, which were significantly higher than by leaf tissue culture ($P < 0.01$). Callus induction frequencies of winter genotypes exceeded those of spring genotypes, but not significantly. Frequencies for embryogenesis were 55.3% in embryo culture, 37.3% in inflorescence culture and 5.0% in leaf tissue culture. Significant genotype × explant interaction in embryogenesis was found ($P < 0.01$).

In cereal crops immature embryo, immature inflorescence and leaf tissue have been commonly used as primary explant in the tissue culture of wheat (Ozias-Atkins and Vasil 1982, Gonzalez et al. 2001), rice (Jyoti and William 1996) and barley (Thomas and Scott 1985, Lührs and Lörz 1987, Timothy et al. 1993, Chang et al. 2003). In rye species, the somatic embryogenesis and plant regeneration from different explant sources of immature embryo (Lu et al. 1984, Zimny and Lörz 1989, Popelka and Altpeter 2001), immature inflorescence (Linacero and Vazquez 1990, Rakoczy-Trojanowska and Malepszy 1993) and leaf tissue culture have been investigated. Immature embryo culture gave the best result, although other factors may have influenced the results. In our study, the highest embryogenesis frequency was from immature embryo culture, being in agreement with previous studies. The lowest frequency was from leaf tissue culture, which was much lower than that reported by Linacero and Vazquez (1986). This result is likely explained by the different genotypes used in the experiments. In this study, interaction of genotype × explant played an important role in the somatic embryogenesis of rye. A similar interaction has been found in barley (Ruiz et al. 1992).

Genotypic effects in embryo culture of rye

Table 8 shows that genotype significantly influenced somatic embryogenesis ($P < 0.05$) and green plant regeneration ($P < 0.001$). No significant differences for embryogenesis and regeneration fre-

Table 6. Effects of ecotypes, genotypes and cold pre-treatment on green plant regeneration (green plants per 100 calli) in rye embryo culture. 190-2 medium with 3% (w/v) sucrose, 3 mg l-1 BA and 0.45% Phytagel was used for regeneration medium. Each treatment contained five replicates.

Eco-type	Genotype	Cold pre-treatment (weeks)					
		0	1	2	3	4	5
Spring	ME8008	51.22	67.22	73.96	60.48	52.63	49.50
	Florida dwarf	65.00	73.96	69.09	67.78	67.95	60.45
	Auvinen	46.00	58.96	59.78	42.31	40.13	33.00
	Average	54.07B	66.71A	67.61A	56.86B	53.57B	47.65B
Winter	Zulpan	67.32	76.50	83.13	71.25	62.71	60.70
	Amilo	68.79	76.67	70.34	66.81	65.00	62.37
	Jussi	48.69	64.62	68.29	50.37	48.49	44.19
	Anna	59.38	66.67	60.19	55.0	46.67	44.13
	Riihi	43.39	52.11	57.85	47.59	43.69	34.80
	Vågones	57.24	61.67	66.86	52.86	50.00	50.00
	Wheeler	57.35	60.25	56.34	50.00	43.93	42.17
	Voima	48.50	53.70	63.64	52.44	50.60	49.66
	Average	56.33B	64.02A	65.83A	55.79B	51.39BC	48.50C
	Total average	55.72B	64.76A	66.32A	56.08B	51.98BC	48.27C

LSD0.05 = 9.80 between cold treatments within spring genotypes
LSD0.05 = 5.85 between cold treatments within winter genotypes
LSD0.05 = 5.01 between cold treatments

Vol. 13 (2004): 363–377.

quencies were found between winter and spring genotype samples. Frequencies of embryogenesis and regeneration varied from 43.30% (Lanzhou) to 70.53 % (Zulpan), and 34.29% (EM-1) to 71.08% (Zulpan), respectively. A significant ($P < 0.01$) correlation between embryogenesis frequency and regeneration frequency ($r = 0.534$) was found.

Genotype is an important influencing factor in the plant tissue culture of cereal crops. In most species of cereals, genetic transformation is strongly dependent on genotype and efficiency is largely determined by culture ability of the tissue (Maddock et al. 1983, Fennel et al. 1996). In the anther and microspore culture of rice (Gosal et al. 1997), wheat (Hu 1997) and rye (Guo and Pulli 2000), culture abilities are genetically controlled and culture efficiencies are genotype dependent. In the immature embryo and inflorescence culture of wheat (Felföldi and Purnhauser 1992, Machii et al. 1998, Gonzalez et al. 2001), rice (Jyoti and William 1996) and barley (Lührs and Lörz 1987). The genotypic effects on somatic embryogenesis and plant regeneration have also been noticed. For barley, somatic embryogenesis and plant regeneration are two independent processes, and are controlled by independent genetic systems (Komatsuda et al. 1989, Stirn et al. 1995). In hexaploid wheat, the day length-sensitive allele *ppd1* play major role in somatic embryogenesis and plant regeneration (Ben et al. 1992). Rye is a crossing-pollinating species, and has a great variability due to its allogamous character. Even plants from the same genotype were not exactly genetically identical, but plants from same genotype are genetically more homogeneous than plants from different genotypes. Different genotypes could show different responses in tissue culture (Linacero and Vazquez 1990). Rakoczy-Trojanowska and Malepszy (1995) sug-

Table 7. Differences between plant organs in rye somatic embryogenesis. AA medium with 3% sucrose, 4 mg l-1 Dicamba and 0.3% Phytagel was used for induction medium. Each treatment contained five replicates.

Eco-type	Genotype	Embryogenic calli per explants (%)		
		Embryo	Inflorescence	Leaf segment
Spring	ME80083	67.3	36.4	3.0
	Florida dwarf	66.6	41.2	2.4
	Vågones vårrug	57.8	43.3	2.1
	Gansu	54.1	40.0	3.3
	Auvinen	53.7	33.4	5.8
	OD	51.1	27.2	4.0
	Rogo	50.9	32.5	6.2
	Kalhek K131	47.5	31.7	5.6
	Floride 401	35.6	33.6	5.4
	Average	53.84A	35.48B	4.20C
Winter	Zulpan	73.2	44.7	3.9
	Amilo	63.5	39.5	7.7
	Jussi	59.4	43.7	6.3
	Anna	60.4	41.5	4.5
	Riihi	45.4	44.1	4.5
	Vågones	51.7	39.7	6.4
	Wheeler	50.6	25.7	10
	Voima	51.2	36.4	4.6
	Average	56.93A	39.41B	5.99C
	Total average	55.29A	37.33B	5.00C

LSD0.05 = 6.23 between plant organs of spring genotypes
LSD0.05 = 6.65 between plant organs of winter genotypes
LSD0.05 = 4.41 between plant organs

gested that *in vitro* response of rye seems to be a complex trait controlled by many genes co-operating in different ways, and the regeneration ability were determined by recessive genes.

In the present study on the embryo culture of rye, both embryogenesis and regeneration differed significantly among genotypes, thus proving the importance of genotype. Genotypic effects also have been observed in inflorescence culture of rye (Linacero and Vazquez 1990, Rakoczy-Trojanowska and Malepszy 1993). A correlation between embryogenesis and regeneration for all genotypes tested in the present study was observed. The genotypes with high embryogenesis also showed comparatively high regeneration. There was no difference in embryogenesis and regeneration between winter and spring genotype groups. Similar results were also demonstrated in embryo,

Table 8. Response of genotypes to embryogenesis and plant regeneration in immature embryo culture of rye. AA medium with 3% sucrose, 4 mg l-1 Dicamba and 0.3% Phytagel was used for induction medium. 190-2 medium with 3% (w/v) sucrose, 3 mg l-1 BA and 0.45% Phytagel was used for regeneration medium. Each treatment contained five replicates.

Eco-type	Genotype	No. of immature embryos	Embryogenic calli (% of embryos)	Green plants (% of embryogenic calli)
Spring	ME-80083	69	66.42	60.14
	KVL-7002	73	63.50	44.32
	Florida dwarf	75	61.00	65.57
	Vågones vårrug	72	60.96	50.00
	Auvinen	65	55.10	51.55
	OD	72	52.53	66.77
	Gansu	69	54.40	56.82
	Rogo	59	54.14	58.62
	Kalhek K131	69	49.00	41.30
	Florida 401	61	47.27	50.40
	Average	68.4	56.43	54.55
Winter	Zulpan	72	70.53	71.08
	Amilo	70	66.90	70.94
	Jussi	70	64.60	56.11
	Bonel	74	59.90	48.17
	Anna	70	58.10	57.14
	Riihi	65	57.85	50.00
	EM-1	61	56.50	34.29
	Elvi	70	55.50	41.50
	Vågones	54	54.59	59.89
	Wheeler	71	53.90	54.30
	Akusti	71	53.33	56.50
	Voima	61	50.60	55.89
	Danko	68	50.00	54.70
	Sangaste	52	47.19	40.77
	Vambo	61	45.27	42.80
	Bylina	66	43.75	38.90
	Lanzhou	68	43.30	48.20
	Average	66.1	54.81	51.83
	Total average	66.96	55.42	52.84

LSD0.05 = 14.93 between spring genotypes for embryogenic callus induction
LSD0.05 = 14.96 between spring genotypes for green plant regeneration
LSD0.05 = 15.21 between winter genotypes for embryogenic callus induction
LSD0.05 = 14.95 between winter genotypes for green plant regeneration
LSD0.05 = 14.92 between genotypes for embryogenic callus induction
LSD0.05 = 14.77 between genotypes for green plant regeneration

Vol. 13 (2004): 363–377.

inflorescence and scutellum culture of wheat and barely (Felföldi and Purnhauser 1992, Fransisco et al. 1999).

Effects of developmental stage of immature embryos on somatic embryogenesis

Influence of embryo developmental stages (stage 1–6 of Zimny and Lörz 1989, size ranging from under 0.5 mm to 3 mm) were tested in this study. Embryo developmental stages significantly ($P < 0.001$) influenced the embryogenic induction (Table 9). Significant highest embryogenesis (64.29%) was obtained from embryo sizes between 0.5–1 mm (about stage 3 of Zimny and Lörz 1989, Table 9).

Embryo size, which is an indicator for the developmental embryonic stage, was a major influencing factor in previous studies on the somatic embryogenesis of wheat, barley and rye. Previous studies have suggested optimal embryo sizes of 1.0–1.5 mm (Redway 1990), 0.5–2 mm (Özgen et al. 1996) and 1.7–1.9 mm (Maes et al. 1996) in wheat, 0.7–1.4 mm (Lührs and Lörz 1987), 1.1–1.5 mm (Ruiz et al. 1992), 1.5–2 mm (Timothy et al. 1993) and 0.5–1.5 mm (Chang et al. 2003) in barley, 1–2 mm (Krumbiegel-Schroeren et al. 1984) in rye. Our results showed an embryo size of 0.5–1 mm (about stage 3 of Zimny and Lörz 1989) to be optimal for somatic embryogenesis. Smaller embryos (size < 0.5 mm, stage 1–2 of Zimny and Lörz 1989) responded to callus induction at a lower rate, while larger embryos (> 2mm, stages 5–6 of Zimny and Lörz 1989) germinated at faster

Table 9. Effects of ecotypes, genotypes and embryo size on somatic embryogenesis (embryogenic calli per 100 embryos) in rye embryo culture. AA medium with 3% sucrose, 4 mg l-1 Dicamba and 0.3% Phytagel was used for induction medium. Each treatment contained five replicates.

Eco-type	Genotype	Embryo Size (1–6 stages of Zimny and Lörz 1989)			
		< 0.5 mm Stage 1–2	0.5–1 mm Stage 3	1–2 mm Stage 4	2–3 mm Stage 5–6
Spring	ME80083	43.33	68.75	59.74	47.37
	Florida dwarf	56.25	68.39	61.86	54.40
	Vågones vårrug	54.86	69.44	66.09	42.74
	Auvinen	57.86	67.14	55.68	44.23
	OD	43.16	56.30	48.83	44.58
	Gansu	47.06	57.72	51.11	49.43
	Rogo	44.85	59.18	53.70	51.06
	Average	49.62C	63.85A	56.72B	47.69C
Winter	Zulpan	64.15	77.94	75.92	58.46
	Amilo	61.54	78.86	71.03	53.23
	Jussi	44.17	64.46	58.76	51.25
	Bonel	56.93	60.75	61.47	57.01
	Anna	63.16	71.34	64.74	48.91
	Riihi	50.63	54.13	53.61	41.27
	Vagones	46.67	56.94	50.26	46.51
	Wheeler	46.83	61.46	55.90	52.46
	Voima	50.41	55.92	53.08	46.87
	Average	53.83B	64.64A	60.53A	50.66B
	Total average	51.99C	64.29A	58.86B	49.36C

LSD0.05 = 5.74 between embryo size within spring genotypes
LSD0.05 = 5.35 between embryo size within winter genotypes
LSD0.05 = 3.90 between embryo size

rate and a higher percentage. These results were in agreement with of Krumbiegel-Schroeren et al. (1984), Zimny and Lörz (1989) in the embryo culture of rye.

Maintenance of morphological competence

Embryogenic calli were subcultured and green plant regeneration abilities were tested at each subculture. Regeneration ability significantly ($P < 0.001$) decreased with increasing number of subcultures. With the exception of 'Zulpan' (19.4%) and 'Vågones' (11.2%), regeneration abilities of all genotypes were under 10% after 8 months of subculture (Table 10).

In cereal crops, embryogenic calli are suitable target tissues for genetic transformation, also being suitable starting materials for cell suspension and protoplast cultures. Immature embryo derived calli have been frequently used for establishment of embryogenic cell suspension used as a source of totipotent protoplasts (Maddock 1987, Ahmed and Sagi 1993) and target tissue for genetic transformation (Becker et al. 1994, Dong et al. 2001). Maintenance of embryogenic capability and regeneration potential has been a critical problem in an efficient *in vitro* culture system (Lührs and Lörz 1987, Chang et al. 2003). Prolonged duration of subculture, has caused the gradual loss of embryogenic competence and regeneration potential (Bregitzer 1991, Jimenez and Bangerth 2001). Somatic embryogenesis and plant regeneration are genetically controlled, and the frequency of embryogenesis and regeneration is genotype dependent. Medium composition and physiological state of the donor plant affects the reaction of the explant under *in vitro* conditions (Lührs and Lörz 1987, Castillo 1998). Embryogenicity and regeneration ability has been maintained for 17 months in barley (Kachhwaha 1997), 36 months in wheat (Varshney 1996) and 34 months in rice (Utomo 1995). In our study, regeneration ability of embryogenic callus of rye was maintained for a maximum of 8 months for subculture. After 9 months subculture, regeneration ability was nearly lost. All green plants regenerated were normal in morphology and seed set.

Acknowledgements. We thank Mr. Jukka Karhu, Ms. Mervi Ylämäki, Ms. Marja Piirainen and Ms. Lioudmila Choumskaia for technical assistance, and the staff of the Laboratory of Plant Physiology and Molecular Biology for technical assistance. We thank Ms. Randi Kumpulainen for the English revision of the manuscript. This work was supported by the Finnish Ministry of Agriculture and Forestry.

Table 10. Influence of subculture age on green plant regeneration (green plants per 100 calli) in rye embryo culture. AA medium with 3% sucrose, 2 mg l-1 2,4-D and 0.3% Phytagel was used as culture medium for embryogenic callus maintenance. Each treatment contained five replicates.

Genotype	Culture age (month)								
	1	2	3	4	5	6	7	8	9
Auvinen (sp)	53.7	50.4	44.1	37.6	34.6	27.3	16.4	11.5	6.8
Zulpan	74.3	68.8	59.3	50.3	46.5	38.6	32.5	26.3	19.4
Amilo	65.4	57.5	52.6	42.5	39.5	29.5	14.6	9.7	2.6
Jussi	56.8	53.7	49.2	41.4	33.6	24.4	11.3	7.6	1.7
Riihi	53.5	49.4	40.3	35.4	30.3	22.3	13.2	8.7	7.7
Vågones	62.4	59.4	51.5	45.3	38.7	33.3	22.7	18.4	11.2
Wheeler	56.7	51.9	41.4	34.2	28.2	23.6	19.4	13.3	5.8
Voima	58.1	50.3	45.4	38.4	33.0	23.5	18.9	12.6	8.4
Average	60.1	55.2	48.0	40.6	35.6	27.8	18.6	13.5	7.9

LSD0.05 = 4.34 between culture ages
sp: spring genotype

Vol. 13 (2004): 363–377.

References

Ahmed, K.Z. & Sagi, F. 1993. Culture of and fertile plant regeneration from regenerable embryogenic suspension cell-derived protoplasts of wheat (*Triticum aestivum* L.). *Plant Cell Reports* 12: 175–179.

Altpeter, F., Popelka, J.C. & Wieser, H. 2004. Stable expression of 1Dx5 and 1Dy10 high-molecular-weight glutenin subunit genes in transgenic rye drastically increases the polymeric glutelin fraction in rye flour. *Plant Molecular Biology* 54: 783–792.

Becker, D., Brettschneider, R. & Lörz, H. 1994. Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. *Plant Journal* 5: 299–307.

Ben Amer, I.M., Worland, A.J. & Börner A. 1992. *In vitro* culture variation of wheat and rye caused by genes affecting plant growth habit *in vivo*. *Euphytica* 61: 233–240.

Bregitzer, P. 1991. Plant regeneration and callus type in barley: effects of genotypes and culture medium. *Crop Science* 32: 1108–1112.

Castillo, A.M., Egana, B., Sanz, J.M. & Cistue, L. 1998. Somatic embryogenesis and regeneration from barley cultivars grown in Spain. *Plant Cell Reports* 17: 902–906.

Castillo, A.M., Vasil, V. & Vasil, I.K. 1994. Rapid production of fertile transgenic plants of rye (*Secale cereale* L.). *Bio/Technology* 12: 1366–1371.

Chang, Y., Zitzewitz, J.V., Hayes, P.M. & Chen, T.H.H. 2003. High frequency plant regeneration from immature embryos of an elite barley cultivar (*Hordeum vulgare* L. cv. Morex). *Plant Cell Reports* 21: 733–738.

Dong, J., Kharb, P., Teng, W. & Hall, T.C. 2001. Characterization of rice transformed via an *Agrobacterium*-mediated inflorescence approach. *Molecular-Breeding* 7: 187–194.

Felföldi, K. & Purnhauser, L. 1992. Induction of regeneration callus cultures from immature embryos of 44 wheat and 3 triticale cultivars. *Cereal Research Communications* 20: 273–276.

Fennell, S., Bohorova, N., Ginkel, M., Crossa, J. & Hoisington, D. 1996. Plant regeneration from immature embryos of 48 elite CIMMYT bread wheats. *Theoretical and Applied genetics* 92: 163–169.

Flehinghaus, T., Deimling, S. & Geiger, H.H. 1991. Methodical improvements in rye anther culture. *Plant Cell Reports* 10: 397–400.

Francisco, B., Martin, A., Lazzeri, P.A. & Barcelo, P. 1999. Medium optimisation for efficient somatic embryogenesis and plant regeneration from immature inflorescences and immature scutella of elite cultivars of wheat, barley and tritordeum. *Euphytica* 108: 161–167.

Gamborg, O.L., Miller, R.A. & Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* 50: 151–158.

Gonzalez, J.M., Friero, E. & Jouve, N. 2001. Influence of genotype and culture medium on callus formation and plant regeneration from immature embryos of *Triticum aestivum* Desf. Cultivars. *Plant Breeding* 120: 513–517.

Gosal, S.S., Sindhu, A.S., Sandhu, J.S., Sandhu-Gill, R., Singh, B., Khehra, G.S. & Dhaliwal, H.S. 1997. Haploid in rice. In: Jain, S.M. et al. (eds.). *In vitro haploid production in higher plants*. Kluwer Academic Publishers, Nertherland. Vol 4. p. 1–35.

Guo, Y.D. & Pulli, S. 2000. Isolated microspore culture and plant regeneration in rye (*Secale cereale* L.). *Plant Cell Reports* 19: 875–880.

Hu, H. 1997. In vitro induced haploids in wheat. In: Jain S.M. et al. (eds.). *In vitro haploid production in higher plants*. Kluwer Academic Publishers, Nertherland. Vol 4. p. 73–97.

Immonen, A.S.T. 1996 Influence of medium and growth regulators on somatic embryogenesis and plant regeneration for production of primary triticales. *Plant Cell, Tissue and Organ Culture* 44: 45–52.

Jimenez, V.M. & Bangerth, F. 2001. Endogenous hormone concentrations and embryogenic callus development in wheat. *Plant Cell, Tissue and Organ Culture* 67: 37–46.

Jyoti, R.R. & William, J.L. 1996. Characterization and manipulation of embryogenic response from in-vitro-cultured immature inflorescences of rice (*Oryza sativa* L.). *Planta* 198: 127–138.

Kachhwaha, S. Varshney, A. & Kothari, S.L. 1997 Somatic embryogenesis and long term high plant regeneration from barley (*Hordeum vulgare* L.) using picloram. *Cereal Research Communications* 25: 117–126.

Kohlenbach, H. & Wernicke, W. 1978. Investigations on the inhibitory effect of agar and function of active carbon in anther culture. *Zeitschrift für Pflanzenphysiologie* 86: 463–472.

Komatsuda, T., Enomoto, S. & Nakajima, K. 1989. Genetics of callus proliferation and shoot differentiation in barley. *Journal of Heredity* 80: 345–350.

Krumbiegel-Schroeren, G., Finger, J., Schroeren, V. & Binding H. 1984. Embroid formation and plant regeneration from callus of *Secale cereale*. *Zeitschrift für Pflanzenzüchtung* 92: 89–94.

Kuhlmann, V. & Foroughi-Wehr, B. 1989. Production of doubled haploid lines in frequencies sufficient for barely breeding programs. *Plant Cell Reports* 8: 78–81.

Li, Z., Xia, G. & Chen, H. 1992. Somatic embryogenesis and plant regeneration from protoplasts isolated from embryogenic cell suspensions of wheat (*Triticum aestivum* L.) *Plant Cell, Tissue Organ Culture* 28: 79–85.

Linacero, R. & Vazquez, A.M. 1986. Somatic embryogenesis and plant regeneration from leaf tissues of rye (*Secale cereale* L.). *Plant Science* 44: 219–222.

Linacero, R. & Vazquez, A.M. 1990. Somatic embryogenesis from immature inflorescences of rye. *Plant Science* 72: 253–258.

Lörz, H., Gobel, E. & Brown, P. 1988. Advance in tissue culture and progress towards genetis transformation of cereals. *Plant Breeding* 100: 1–25.

Lu Chin-Yi, Chandler, S.F. & Vasil, I.K. 1984. Somatic embryogenesis and plant regeneration from cultured immature embryos of rye (*Secale cereale* L.). *Journal of Plant Physiology* 115: 237–244.

Lührs, R. & Lörz, H.1987. Plant regeneration in vitro from embryogenic cultures of spring- and winter- type barley

(*Hordeum vulgare* L.) varieties. *Theoretical and Applied Genetics* 75: 16–25.

Machii, H., Mizuno, H., Hirabayashi, T., Li, H. & Hagio, T. 1998. Screening wheat genotypes for high callus induction and regeneration capability from anther and immature embryo cultures. *Plant Cell, Tissue and Organ Culture* 53: 67–74.

Maddock, S.E. 1987. Suspension and protoplast culture of hexaploid wheat (*Triticum aestivum* L.). *Plant Cell Reports* 6: 23–26.

Maddock, S.E., Lancaster, V.A., Risiott, R. & Franklin, J. 1983. Plant regeneration from cultured immature embryos and inflorescences of 25 cultivars of wheat (*Triticum aestivum* L.). *Journal of Experimental Botany* 34: 915–926.

Maes, O.C., Chibbar, R.N., Caswell, K., Leung, N. & Kartha, K.K. 1996. Somatic embryogenesis from isolated scutella of wheat: effects of physical, physiological and genetic factors. *Plant Science* 121: 75–84.

Moieni, A., Lokos-Toth, K. & Sarrafi, A. 1997. Evidence for genetic and media effect on haploid regeneration in the anther culture of hexaploid wheat (*Triticum aestivum* L.). *Plant Breeding* 116: 502–504.

Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum* 15: 473–497.

Müller, A.G. & Grafe, R. 1978. Isolation and characterization of cell lines of *Nicotiana tabacum* lacking nitrate reductase. *Mol Gen Genet* 161: 67–76.

Özgen, M., Turet, M., Özcan, S. & Sancak. 1996. Callus induction and plant regeneration from immature and mature embryos of winter and durum wheat genotypes. *Plant Breeding* 115: 455–458.

Ozias-Atkins, P. & Vasil, I.K. 1982. Plant regeneration from cultured immature embryos and inflorescences of *Triticum aestivum* L. (wheat): evidence for somatic embryogenesis. *Protoplasm* 110: 95–105.

Popelka, J.C. & Altpeter, F. 2001. Interactions between genotypes and culture media components for improved in vitro response of rye (*Secale cereale* L.) inbred lines. *Plant Cell Reports* 20: 575–582.

Popelka, J.C. & Altpeter, F. 2003a. *Agrobacterium tumefaciens*- mediated genetic transformation of rye (*Secale cereale* L.). *Molecular Breeding* 11: 203–211.

Popelka, J.C. & Altpeter, F. 2003b. Evaluation of rye (*Secale cereale* L.) inbred lines and their crosses for tissue culture response and stable genetic transformation of homozygous rye inbred line L22 by biolistic gene transfer. *Theoretical and Applied genetics* 107: 583–590.

Potrykus, L., Harms, C.T. & Lörz, H. 1979. Callus formation from cell culture protoplasts of corn (*Zea Mays* L.). *Theoretical and Applied Genetics* 54: 209–214.

Pragya, V., Chowdhury, V.K., Wu, R. & Jain, R.K. 2002. Gus expression as a novel system for optimization of *Agrobacterium*-mediated transformation procedure in a Basmati rice variety, Pusa Basmati 1. *Physiology and Molecular Biology of Plants* 8: 141–147.

Rakoczy-Trojanowska, M. & Malepszy, S. 1993. Genetic factors influencing regeneration ability in rye (*Secale cereale* L.) I. Immature inflorescences. *Theoretical and Applied Genetics* 86: 406–410.

Rakoczy-Trojanowska, M. & Malepszy, S. 1995. Genetic factors influencing the regeneration ability of (*Secale cereale* L.). II Immature embryos. *Euphytica* 83: 233–239.

Redeway, F.A., Vasil, V., Lu, D. & Vasil, I.K. 1990 Identification of callus types for long-term maintenance and regeneration from commercial cultivars of wheat (*Triticum aestivum* L.) *Teoretical and Applied Genetics* 79: 609–617.

Ruiz, M.L., Rueda, J., Pelaez, M.I., Espino, M., Candela, E., Sendino, A.M. & Vazquez, A.M. 1992. Somatic embryogenesis, plant regeneration and somaclonal variation in barley. *Plant Cell, Tissue and Organ Culture* 28: 97–101.

Rybczynski, J.J. 1979. The influence of cytokinins (BAP, KIN, ZEAT) on the processes of callusing and caulogenesis of the immature embryo scutellum of di-and tetraploid rye (*Secale cereale* L.). *Genetic Polonica* 20: 11–23.

Stirn, S., Mordhorst, A.P., Fuchs, S. & Lörz, H. 1995. Molecular and biochemical markers for embryogenic potential and regenerative capacity of barley (*Hordeum vulgare* L.) cell cultures. *Plant Sciences* 106: 195–206.

Thomas, E., Hoffmann, F. & Wenzel, G. 1975. Haploid plantlets from microspores of rye. *Zeitschrift für Pflanzenzüchtung* 75: 106–113.

Thomas, M.R. & Scott, K.J. 1985. Plant regeneration by somatic embryogenesis from callus initiated from immature embryos and immature influence of *Hordeum vulgare* L. *Journal of Plant Physiology* 121: 159–169.

Timothy, J.G., Babczinsky, J. & Kook, H. 1993. Regeneration from intact and sectioned immature embryos of barley (*Hordeum vulgare* L.): scutellum exhibits an apico- basal gradient of embryogenic capacity. *Plant Science* 90: 211–218.

Trifonova, A., Madsen, S. & Olesen, A. 2001. *Agrobacterium*-mediated transgene delivery and integration into barley under a range of in vitro culture conditions. *Plant Science* 161: 871–880.

Utomo, H.S., Croughan, S.S. & Croughan, T.P. 1995. Suspension and protoplast culture of U.S. rice cultivars. *Plant Cell Reports* 15: 34–37.

Varshney, A., Kant, T., Sharma, V.K., Rao, A. & Kothari, S.L. 1996. High frequency plant regeneration from immature embryo cultures of *Triticum aestivum* L. and *T. durum*. *Cereal Research Communications* V (24) 4: 409–416.

Vasil, V., Srivastava, V., Castillo, A.M., From, M.E. & Vasil, I.K. 1993. Rapid production of transgenic wheat plants by direct bombardment of cultured embryos. *Bio/Technology* 11: 1553–1558.

Wang, X. & Hu, H. 1984. The effect of potato II medium for Triticale anther culture. *Plant Science Letter* 36: 237–239.

Wan, Y. & Lemaux, P.G. 1994. Generation of large numbers of independently transformed fertile barley plants. *Plant Physiology* 104: 37–48.

Whitney, P.J. 1996 Hormone indepedent root organ cultures of rye (*Secale cereale* L.). *Plant Cell, Tissue and Organ Culture* 46: 109–115.

Yin, Y., Shizhong, L., Yiming, C., Hongqi, G., Wengzhong, T., Ying, C. & Liangcai, L. 1993. Fertile plants regener-

Vol. 13 (2004): 363–377.

ated from suspension culture-derived protoplasts of an indica type rice (*Oryza sativa* L.). *Plant Cell, Tissue and Organ culture 32*: 61–68.

Zhang, L., Rybcznski, J.J., Langenberg, W.G., Mitra, A. & French, R. 2000. An efficient wheat transformation procedure: transformed calli with long-term morphogenic potential for plant regeneration. *Plant Cell Reports* 19: 241–250.

Zimny, J. & Lörz, H. 1989. High frequency of somatic embryogenesis and plant regeneration of rye (*Secale cereale* L.). *Plant Breeding* 102: 89–100.