Callus induction and plant regeneration from immature and mature embryos and immature inflorescences of eight Croatian winter wheat cultivars (*Triticum aestivum* L.)

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Kallusbildung und Regeneration von Pflanzen aus unreifen und reifen Embryonen und Blütenständen bei acht kroatischen Winterweizensorten (*Triticum aestivum* L.)

1. Introduction

The genetic transformation of plants in vitro is largely dependent on the ability of tissue to develop somatic callus and to regenerate whole plants. In wheat, different explants have been used for somatic callus induction: immature embryos (MADDOCK, 1985; MATHIAS and SIMPSON, 1986; CHOWDHURY et al., 1991; FENNELL et al., 1996; VARSHNEY and ALTPETER, 2001) or immature scutella (BARRO et al., 1999), immature leaves (AHUJA et al., 1982; ZAMORA and SCOTT, 1983), immature inflorescences (GOSCH-WACKERLE et al., 1979; OZIAS-AKINS and VASIL, 1982; MADDOCK et al., 1983; EAPEN and RAO, 1985; BARRO et al., 1999), mature embryos (OZIAS-AKINS and VASIL, 1983; KATO et al., 1991; ÖZGEN et al., 1996), mesocotyls (YURKOVA et al., 1982), seeds (GOSCH-WACKERLE et al., 1979) and apical meristems (MCHUGEN, 1983). Wheat immature embryos and scutella in most instances have proved to be the most efficient sources for regenerating whole plants not only in bread wheat (MADDOCK et al., 1983, REDWAY et al., 1990, BARRO et al., 1999), but also in durum wheat (HE and LAZZERI, 2001). However, using a slightly modified version of the endosperm-supported callus induction method of BARTÓK and SÁGI (1990), ÖZGEN et al. (1996) found that regeneration capacity of some durum wheat genotypes might be greater when using mature rather than immature embryos. Similarly, BARRO et al. (1999) found that regeneration capacity from immature inflorescences of some wheat genotypes is comparable to the regeneration from immature scutella.

Callus induction and regeneration from immature and mature embryos and immature inflorescences have proved to be genotype-dependent and strongly influenced by the components of the media used (SEARS and DECKARD, 1982;

Zusammenfassung

Acht kroatische Winterweizensorten (*Triticum aestivum* L.) wurden auf ihre Fähigkeit untersucht, Kallus zu bilden und ganze Pflanzen aus unreifen und reifen Embryonen und unreifen Blütenständen zu regenerieren. Der höchste durchschnittliche Prozentsatz der Regeneration bei den Weizensorten (21.6 %) konnte erreicht werden, als unreife Embryonen als Explantate verwendet wurden, wobei die höchste Regenerationsrate die Sorten Žitarka (57 %) und Edita (54 %) erreichten. Manche Sorten regenerierten sich jedoch ebenso effizient (Edita), und noch besser (Lipa), als unreife Blütenstände eingesetzt wurden. Die größte Regenerationsfähigkeit aus reifen Embryonen (26 % bei Sorte Magdalen) war viel niedriger als die höchste Regenerationsfähigkeit aus unreifen Embryonen oder Blütenständekulturen. Was die Medien mit einer 2,4-D Lösung betrifft, die für die Kallusbildung aus unreifen Embryonen gebraucht wurden, war die Pflanzenregeneration erheblich besser als im Medium mit Picloram. Im Gegensatz dazu konnte bei unreifen Blütenständen das beste Ergebnis durch die Anwendung von einem picloramhaltigen Medium erreicht werden.

Schlagworte: Winterweizen, Embryokultur, unreife Blütenstände, Pflanzenregeneration.

Summary

Eight Croatian winter wheat cultivars (*Triticum aestivum* L.) were evaluated for their ability to produce callus and to regenerate whole plants from immature and mature embryos and immature inflorescences. The highest mean percentage regeneration across cultivars (21.6 %) was obtained when immature embryos were used as explant with the highest regeneration of cultivar Žitarka (57 %) and Edita (54 %). However, some cultivars regenerated equally efficiently (Edita) or better (Lipa) when immature inflorescences were used. The highest regeneration capacity from mature embryos (26 % for cultivar Magdalen) was much below the best regeneration capacities from the immature embryo or inflorescence culture. As far as the media provided with 2,4-D were used for callus induction from immature embryos, plant regeneration was significantly better than in a medium with picloram. With immature inflorescences, on the contrary, the best culture response was obtained using medium containing picloram.

Key words: winter wheat, embryo culture, immature inflorescences, plant regeneration.

FENNELL et al., 1996; ÖZGEN et al., 1996; BARRO et al., 1999). Therefore, the present investigation was undertaken to examine callus induction and plant regeneration from immature and mature embryos and immature inflorescences of eight winter wheat cultivars in order to define suitable explants sources and cultivars with the best regeneration and to establish optimum media for plant regeneration.

2. Material and methods

Eight Croatian cultivars (Kuna, Banica, Lipa, Magdalen, Žitarka, Edita, Hana, Barbara) of winter wheat (*Triticum aestivum* L.) were grown in the field and used to collect immature and mature embryos and immature inflorescences.

Immature seeds, 14-18 days after anthesis, were surface sterilized with 70 % ethanol for 90 sec and 1.5 % sodium hypochlorite solution with 0.1 % Tween 20 for 18 min, followed by four changes of sterile distilled water. Immature embryos 0.5-1.5 mm in size were aseptically isolated from seeds under a stereo dissecting microscope and plated with the scutellum exposed onto three modified MS media (MURASHIGE and SKOOG, 1962) MS1, MS2 and MS3 (table 1). Embryos were reared in disposable plastic Petri dishes (90 x 15 mm). All media were solidified with 0.25 % Gerlite and the pH adjusted to 5.8 prior to autoclaving. The cultures were incubated at 26 °C in the dark for 3 weeks. For shoot initiation, calli were transferred to media in which 2,4-D concentration was reduced to 0.2 mg/l (additionally in MS2 picloram was omitted) and incubated at 26 °C with 16/8 h light/dark photoperiod.

Mature seeds (three months after harvest ripeness) were surface-sterilized for 5 min in 90 % ethanol and rinsed three times in sterile distilled water. Seeds were then imbibed in sterile water at room temperature for 3 h, sterilized again for 30 min in 6.5 % sodium hypochlorite with 0.1 % Tween 20, rinsed with four changes of sterile distilled water and imbibed in sterile water for 12 h at 26 °C. For callus induction from mature embryos, the endosperm-supported callus induction method was used (AHMED et al., 1992; ÖZGEN et al., 1996). Mature embryos were moved slightly from imbibed seeds with a scalpel but were not excised. The seeds with moved embryos were placed furrow downwards in disposable plastic Petri dishes (90 x 15 mm) containing 7 ml of 2,4-D solution (8 mg/l). Dishes were incubated at

Table 1: Media composition for callus induction from wheat immature embryos and inflorescences
Tabelle 1: Zusammensetzung der Medien für die Kallusbildung aus unreifen Embryonen und Blütenständen von Weizen

Medium	Composition
MS1	MS salts and vitamins (MURASHIGE and SKOOG, 1962) supplemented with 2 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) and 30 g/l sucrose
MS2	MS salts and vitamins supplemented with 0.5 mg/l 2,4-D, 2.2 mg/l picloram (4-amino-3,5,6-trichloropicolinic acid), 100 mg/l ascorbic acid and 30 g/l sucrose
MS3	MS salts and vitamins supplemented with 2 mg/l 2,4-D, 30 g/l sucrose, 100 mg/l casein hydrolysate and 500 mg/l glutamine.
MS4	MS salts and vitamins supplemented with 4 mg/l picloram

26 °C in the dark for 11 days. Developing calli were removed from the seeds and transferred to hormone-free MS medium. The pH of the 2,4-D solution and culture media was adjusted to 5.8 and autoclaved. Callus cultures were incubated at 26 °C in the dark for 3 weeks and grown at 26 °C with 16/8 h light/dark photoperiod.

Tillers containing inflorescences ranging in length from 0.3-1.2 cm were harvested and sterilized according to the method of BARCELO and LAZZERI (1995). Inflorescences were cut into transverse sections (segments) of approx. 1 mm and placed into disposable plastic Petri dishes (90 x 15 mm) containing MS1 or MS4 callus induction medium (table 1). Possible effect of inflorescences length was examined by defining two size-classes: 0.3–0.7 cm (48 segments) and 0.8-1.2 cm (36 segments). The cultures were incubated at 26 °C in the dark for 4 weeks. For regeneration, calli were transferred to MS medium supplemented with 0.1 mg/l 2,4-D, 5 mg/l zeatin and 30 g/l sucrose and incubated at 26 °C with 16/8 h light/dark photoperiod for 7 weeks with the passages to the fresh medium after 3 weeks.

For immature and mature embryos the regeneration potential was assessed after 4 weeks whereas for immature inflorescences after 7 weeks of culture on the shoot initiation medium. Plantlets regenerated from all three different explant sources were cut and transferred to hormone-free MS medium (MS basal salts and vitamins supplemented with 30 g/l sucrose).

The percentage callus induction was defined as the ratio between the number of explants (embryos or inflorescence segments) forming callus and the total number of plated explants multiplied by 100. Regeneration capacity was defined as the ratio between the number of calli producing shoots and the number of plated explants multiplied by 100. Twelve explants per cultivar x medium combination for all three types of explants were placed in each of seven Petri dishes. Analysis of Variance and Duncan's Multiple Range Test were conducted for each explant source separately because of different media used, as well as for all three explant sources included, taking into account only the best performing media for each cultivar x explant combination.

3. Results

3.1 Immature embryo culture

Percentages of callus induction across cultivars and media varied between 88–100 % (table 2). Mean percentage of callus induction was significantly lower when using the MS2 medium (95.5 %) compared to MS1 and MS3 media (98.4 %). Mean percentages of plant regeneration for three media tested were 18.6, 7.8 and 19.3 % for MS1, MS2 and MS3 respectively. On MS2 medium calli of all cultivars except Edita often produced adventive roots that negatively affected plant regeneration. Media MS1 and MS3 were of the same value for latter plant regeneration with exceptions of the cultivars Žitarka and Banica (table 2). Among eight cultivars tested, the best regeneration capacity was displayed by cultivars Žitarka on MS1 medium (57 %) and Edita on MS1 and MS3 media (51 and 54 % respectively). Other cultivars had lower percentage plant regeneration, while Magdalen did not regenerate at all (table 2).

Table 2: Callus induction and regeneration from wheat immature embryos as affected by medium composition and cultivar
Tabelle 2: Kallusbildung und Regeneration aus unreifen Weizenembryonen in Abhängigkeit von Mediumzusammensetzung und Sorte

Cultivar	MS1		MS2		MS3	
	Callus induction (%)	Regeneration (%)	Callus induction (%)	Regeneration (%)	Callus induction (%)	Regeneration (%)
Žitarka	96ab	57a	88c	20c	96ab	38b
Edita	99a	51a	90bc	19c	99a	54a
Hana	94abc	20c	98ab	15c	99a	20c
Barbara	99a	2d	99a	0d	100a	2d
Lipa	100a	16c	100a	6d	96ab	21c
Banica	99a	2d	91bc	2d	100a	18c
Kuna	100a	1d	99a	0d	100a	1d
Magdalen	100a	0d	99a	0d	97a	0d
Mean	98.4A	18.6A	95.5B	7.8B	98.4A	19.3A

Values followed by the same letter are not significantly different at the 0.05 probability level

3.2 Mature embryo culture

Endosperm-supported callus induction from mature embryos varied depending on cultivars from 57 % (cultivar Hana) to 98 % (cultivar Magdalen). The mean percentage callus induction across cultivars was 80.6 % (table 3). The mean percentage plant regeneration across cultivars was only 11.1 %. The best regeneration capacity was displayed by the cultivar Magdalen (26 %).

Table 3: Callus induction in 2,4-D solutions (8 mg/l) and regeneration capacity from wheat mature embryos
Tabelle 3: Kallusbildung in 2,4-D Lösung (8 mg/l) und Regenerationsfähigkeit aus reifen Weizenembryonen

Cultivar	Callus induction (%)	Regeneration (%)
Žitarka	69d	4d
Edita	85bc	13bc
Hana	57e	2d
Barbara	77cd	10bcd
Lipa	91ab	15b
Banica	79bcd	6cd
Kuna	89abc	13bc
Magdalen	98a	26a
Mean	80.6	11.1

Values followed by the same letter are not significantly different at the 0.05 probability level

3.3 Immature inflorescences culture

Callus induction from immature inflorescences across cultivars was slightly but significantly higher on MS1 (89.5 %) than on MS4 medium (84.0 %) (table 4). The common characteristic of the calli was that they were almost all embryogenic. Despite this, a broad range of regeneration capacity across cultivars (0–56 %) was observed (table 4). Mean regeneration capacity did not significantly differ between MS1 and MS4 media (13.1 and 12.3 % respectively).

The best regeneration capacity was observed for cultivars Edita (56 %) after induction on MS4 medium and Lipa (40 %) after induction on MS1 medium. There was no significant difference in regeneration capacity between the segments prepared from inflorescences 0.3–0.7 cm and 0.8–1.2 cm long (data not shown).

3.4 Comparison of three different explant sources for eight wheat cultivars

Generally, the highest mean percentage regeneration across cultivars (21.6 %) was obtained when immature embryos were used as explant (table 5). The highest regeneration capacity showed cultivar Žitarka from immature embryos (57 %) and cultivar Edita from immature embryos and inflorescences (54 % and 56 % respectively) (table 5). Cultivar Lipa had the best regeneration from immature inflorescences (40 %), while cultivar Magdalen that did not regenerate any plants from immature embryos showed the best regeneration from mature embryos (26 %).

4. Discussion

High regeneration capacity of wheat cultivars is an essential prerequisite for improving them by any method of genetic transformation. Regeneration is shown to be greatly dependent on explant (Barro et al., 1999; He and Lazzeri,

Table 4: Callus induction and regeneration capacity from wheat immature inflorescences as affected by medium composition and cultivar
Tabelle 4: Kallusbildung und Regenerationsfähigkeit aus unreifen Blütenständen von Weizen in Abhängigkeit von Mediumzusammensetzung und Sorte

	MS1		MS4	
Cultivar	Callus induction (%)	Regeneration (%)	Callus induction (%)	Regeneration (%)
Žitarka	92ab	10de	87abc	0e
Edita	91ab	23c	92ab	56a
Hana	93ab	9de	86abc	10de
Barbara	79bcd	11de	88abc	1e
Lipa	96a	40b	92ab	18cd
Banica	91ab	6e	86abc	8de
Kuna	82abc	2e	67d	4e
Magdalen	92ab	4e	74cd	1e
Mean	89.5A	13.1A	84.0B	12.3A

Values followed by the same letter are not significantly different at the 0.05 probability level

Table 5: The highest regeneration capacity achieved in optimal callus induction medium for different types of explants and cultivars
Tabelle 5: Höchste im optimalen Kallusbildungsmedium erreichte Regenerationsfähigkeit für unterschiedliche Explantate und Sorten

Cultivar	Immature embryos (%)	Mature embryos (%)	Immature inflorescences (%)
Žitarka	57a	4ghi	10defghi
Edita	54a	13defgh	56a
Hana	20cde	2hi	10defghi
Barbara	2hi	10defghi	11defghi
Lipa	21cd	15cdefg	40b
Banica	18cdef	6fghi	8efghi
Kuna	1hi	13defgh	4ghi
Magdalen	0i	26c	4ghi
Mean	21.6A	11.1C	17.9B

Values followed by the same letter are not significantly different at the 0.05 probability level

2001), genotype and medium used (SEARS and DECKARD, 1982; FENNELL et al., 1996). Consequently, our objective was to examine the immature and mature embryo and immature inflorescence culture response of eight Croatian winter wheat cultivars.

The highest mean percentage regeneration across cultivars (21.6 %) obtained when immature embryos were used as explant in this study is consistent with the results of BARRO at al. (1999) who found that average shoot regeneration from scutella was clearly higher than from inflorescences. However, in our case some cultivars regenerated equally efficiently (Edita) or better (Lipa) when immature inflorescences were used as the explant source. This finding is interesting because it may increase efficiency of gene transfer technology to genotypes that have poor tissue culture response from immature embryos. An additional advantage of using inflorescences is easier explants preparation compared to immature embryos. On the other hand, mature embryos, available without limit at any time, are the least frequently used explants source because of the low frequency of callus induction and plant regeneration. Results obtained in this study confirm findings of ÖZGEN et al. (1996) that mature embryos of some genotypes could regenerate plants better then immature embryos. However, the highest percentage plant regeneration from mature embryos in this study (26 % for cultivar Magdalen) was much below the best regeneration capacity from the immature embryo or inflorescence culture (table 5).

Three cultivars, Žitarka, Edita and Lipa could be distinguished as the cultivars with the best tissue culture performance. Calli initiated from immature embryos of Žitarka and Edita were more compact and embryogenic in comparison to other cultivars and this accounted for the best regeneration capacity of these two cultivars (57 % and 54 % respectively) from immature embryos (table 2). On the contrary, calli obtained from immature inflorescences were all embryogenic, but only cultivars Edita and Lipa showed good regeneration capacity from immature inflorescences (56 % and 40 % respectively).

Media used in this study all consisted of MS basal salts and vitamins (MURASHIGE and SKOOG, 1962), but they differed in the auxin source and some organic supplements (table 1). REDWAY et al. (1990) noticed a positive influence of casein hydrolysate and glutamine on callus formation, but in our study callus formation as well as regeneration were equally efficient on both MS3 medium, containing these supplements, and MS1 medium without them. The composition of MS2 medium in which picloram was used as the main auxin source was identical to that of CM4C (CHENG et al., 1997), except that maltose was replaced by sucrose. Although auxin 2,4-D is the most commonly used exogenous growth regulator added to culture media for cereals, BARRO et al. (1999) reported that picloram gave rise to more regenerative culture when added to induction media in place of 2,4-D. In our experiment with the immature embryos, picloram did not improve culture responses in any of the cultivars on trial. On the contrary, roots appeared frequently and spoiled the percentage plant regeneration on MS2 medium. However, when picloram was used in concentration 4 mg/l (MS4) for culturing immature inflorescences as suggested by BARRO et al. (1999), the cultivar Edita showed a significantly better regeneration capacity in comparison with medium MS1 which had 2,4-D as auxin source.

In conclusion, three cultivars, Edita, Žitarka and Lipa were chosen for subsequent transformation experiments. Edita showed equal regeneration capacity from immature embryos and immature inflorescences, Žitarka had the best

response to immature embryo and Lipa to immature inflorescence culture. Concerning the medium, only the simplest MS1 medium will be used for callus induction from immature embryos in further experiments, whereas for immature inflorescences, at least for cultivar Edita, induction medium containing picloram (MS4) will be used.

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