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BRIEF COMMUNICATION

Regeneration capacity of calli derived from immature embryos in spring barley cultivars

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Abstract

Callogenesis, somatic embryogenesis and regeneration capacity in twenty-three agronomically important spring barley (*Hordeum vulgare* L.) cultivars on induction media with 2,4-dichlorophenoxyacetic acid (2,4-D) or 3,6-dichloro-*o*-anisic acid (dicamba) and on modified regeneration media were studied. The frequency of zygotic embryos exhibiting callogenesis varied from 88 to 100 % according to genotype. Dicamba was more suitable for somatic embryogenesis induction and exhibited a higher frequency of regenerants than did 2,4-D. Green regenerants were obtained in all cultivars, and there were no albino plants. Except for cv. Victor all cultivars used in the experiment showed lower regeneration capacity as compared to the model cv. Golden Promise.

Additional key words: callus induction, dicamba, 2,4-dichlorophenoxyacetic acid, *Hordeum vulgare, in vitro* culture, somatic embryogenesis.

Different explant sources have been used for induction in barley of embryogenic calli and regeneration *via* somatic embryogenesis. In recent studies using explants are mature embryos (Akula *et al*. 1999, Ganeshan *et al*. 2003, Vikrant and Rashid 2003), immature zygotic embryos (Barro *et al.* 1999, Chang *et al.* 2003), immature inflorescences (Barro *et al*. 1999, Havrlentová *et al*. 2001), leaf base/apical meristem (Pasternak *et al*. 1999, Ganeshan *et al*. 2003, Ogburia 2003/4) and fertile green plants (Eudes *et al*. 2003).

In barley (*Hordeum vulgare* L.), the ability of callus induction and regeneration capacity are genotype-dependent (Goldstein and Kronstad 1986) and are also influenced by culture medium composition (Bregitzer 1992, Dahleen 1995, Castillo *et al*. 1998, Jiang *et al*. 1998). Recent studies have been conducted to improve callus quality, to enhance frequency of plant regeneration, and to decrease albinism in recalcitrant barley cultivars (Jiang *et al*. 1998, Cho *et al*. 1998, Chang *et al*. 2003).

Medium composition, including growth regulators, is important factor influencing callus induction and regeneration capacity. Dicamba (3,6-dichloro-*o*-anisic acid) is the most widely used auxin in the induction media in the experiments focused on somatic embryogenesis, plant regeneration and genetic transformation in cereals. The auxin 2,4-D is the most commonly used for callus induction in cereals, especially in wheat (Viertel *et al*. 1997, Harvey *et al*. 1999). However, 2,4-D has been shown to cause a loss of totipotency from the long-term cultured barley callus (Bregitzer *et al*. 1995). The media contained dicamba gave the best embryogenic callus induction, maintenance and regeneration (Castillo *et al.* 1998). Jiang *et al*. (1998) did not observed differences in media with dicamba or 2,4-D in callus-induction frequencies. Barro *et al*. (1999) found out that picloram in the induction medium gave rise to more regenerative cultures than 2,4-D. The best regeneration coefficients for most barley cultivars were obtained after culture on dicamba or dicamba with 2,4-D (Przetakiewicz *et al*. 2003).

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; dicamba - 3,6-dichloro-*o*-anisic acid.
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The aim of our experiments was to evaluate somatic embryogenesis induction and plant regeneration in spring barley cultivars of agronomic importance in order to develop efficient *in vitro* regeneration methods to be used in genetic transformation experiments. The influence of auxins, 2,4-D and dicamba, on the embryogenic callus induction and plant regeneration capacity was investigated.

Twenty-three commercially important two-rowed spring barley cultivars of Czech, Slovak and German origin were used in the experiments. These included 13 malting cultivars (Akcent, Alexis, Amulet, Annabell, Atribut, Granát, Forum, Krona, Nordus, Novum, Olbram, Scarlett, Tolar) and 10 animal feed cultivars (Ditta, Heris, Kompakt, Kosan, Orbit, Orthega, Pejas, Primus, Prosa, Viktor), and a model genotype for tissue culture (Golden Promise). The donor plants were grown in soil and cultivated in a growth chamber at 16-h photoperiod with irradiance of 500 μmol(photon) m^{-2} s^{-1} and under a day/night temperature of 15/12 °C and 70 % humidity. Seeds of cultivars were purchased from the Research Institute of Crop Production Prague-Ruzyně (Czech Republic).

Spikes were collected 12 - 14 d after pollination when the immature zygotic embryos were approximately 1.0 - 1.5 mm in length. Immature caryopses were surface-sterilized for 1 minute in 70 % ethanol followed by two washes in sterile distilled water. They were then stirred for 5 min in 6 % (v/v) sodium hypochlorite, and subsequently rinsed four times with sterile water (after surface sterilization). The embryos were dissected from the immature caryopses and the embryonic axis was removed from each. The embryonic axes were then placed with the scutellum side up on the surface of BCI medium (Wan and Lemaux 1994), which was used for callus induction. It was supplemented with 350 mg dm^{-3} myo-inositol, 690 mg dm^{-3} proline, 1 mg dm^{-3} thiamine HCl, 1 g dm^{-3} casein hydrolysate, 30 g dm^{-3} maltose, 1.25 mg dm^{-3} $CuSO_4$, and solidified with 3.5 g dm^{-3} *Phytagel*. *Phytagel* was autoclaved separately at 121 °C for 20 min. All other components of the medium were filter-sterilized, warmed to approximately 60 °C, and then combined with *Phytagel*. The pH of the medium was adjusted to 5.8 before filter-sterilization.

The induction medium contained either 2.5 mg dm^{-3} dicamba or 2.5 mg dm^{-3} 2,4-D. The induced calli were transferred to a fresh induction medium after 2 weeks. Calli derived from immature embryos were cultured for 4 weeks at 25 °C in the dark. Callus quality was visually assessed under a stereomicroscope 3 - 4 weeks after culture establishment. Callus induction was evaluated as the percentage of zygotic embryos forming calli per number of zygotic embryos planted.

Calli were divided and transferred to FW medium (Harwood *et al*. 1995) containing 20 g dm^{-3} maltose, 1.25 mg dm^{-3} $CuSO_4$, but without alanine and phyto-hormones. The regeneration media were sterilized similarly to the induction media. Explants were cultured on the regeneration media for 4 weeks at 25 °C per 16-h photoperiod as described above. Resulting green plantlets were counted in each plate. The regeneration capacity was assessed as the number of green plants per one hundred cultured zygotic embryos.

Fifty embryos divided in ten plates were cultured of each from 24 genotypes including Golden Promise. The data were analysed by *ANOVA* using Duncan´s multiple-comparison test at the 5 % probability level. Software *NCSS 60* (*NCSS,* Kaysville, USA) was used.

Calli and somatic embryos from immature zygotic embryos were induced on both induction media either with dicamba or with 2,4-D. The average callus induction varied from 64 to 100 % according to 2,4-D or dicamba application. The commercial cultivars did not exhibit any significant difference in frequency of callus induction as compared to Golden Promise (Table 1). Cultivars Amulet, Kosan, Novum, Orbit, Orthega and Viktor showed slightly higher callus induction rates than Golden Promise, but the differences were insignificant.

Table 1. Induced somatic embryogenesis in barley cultivars: calli induced [% of inoculated zygotic embryos], callus quality (+++ - compact, embryogenic, white-coloured callus, ++ - medium quality of callus, + - lower quality of callus, namely soft, friable and root-producing), and number of plant regenerants [per 100 inoculated zygotic embryos]. Means in particular column with different letters are statistically significant at $P = 0.05$.

Cultivars	Calli induced	Callus quality		Number of regenerants
		dicamba	2,4-D	
Golden Promise	98 a	+++	++	401 a
Akcent	96 a	++	++	72 bc
Alexis	94 a	++	++	86 bc
Amulet	100 a	+++	++	148 bc
Annabell	98 a	++	++	126 bc
Atribut	86 a	+++	+++	164 bc
Ditta	90 a	++	+	62 c
Forum	94 a	++	++	156 bc
Granát	98 a	++	++	136 bc
Heris	98 a	++	++	106 bc
Kompakt	80 a	++	++	104 bc
Kosan	100 a	++	++	116 bc
Krona	96 a	+++	++	117 bc
Nordus	88 a	++	++	72 bc
Novum	100 a	+++	++	96 bc
Olbram	92 a	++	++	58 c
Orbit	100 a	+++	++	86 bc
Othega	100 a	++	++	132 bc
Pejas	96 a	++	++	102 bc
Primus	98 a	++	+	64 c
Prosa	98 a	++	++	72 bc
Scarlett	88 a	++	++	100 bc
Tolar	98 a	+	+	72 bc
Victor	100 a	+++	++	294 ab

There were visually observed variations in the quality of formed calli among the genotypes and also between the media supplemented with two types of auxins (Table 1). Calli from cultivars Ditta and Tolar produced large amounts of roots and the calli were highly friable. A compact embryogenic callus was observed more frequency on the induction medium containing dicamba in comparison to that containing 2,4-D.

The study on the effect of the two auxin types on regeneration capacity of the 24 cultivars including Golden Promise showed that the number of green plants per one hundred zygotic embryos was significantly higher when zygotic embryos were induced on BCI medium containing dicamba than when incubated on the medium containing 2,4-D. Mean of plant regeneration on media with dicamba was 146 plants per one hundred zygotic embryos and it was significant different from plant regeneration on induction media with 2,4-D (99 plants per one hundred zygotic embryos). Mean of callus induction per one hundred zygotic embryos on media with dicamba or 2,4-D was the similar (95) and differences were insignificant.

All cultivars produced green regenerants without any albino plants. All commercial cultivars showed a significantly lower plant regeneration rate than did Golden Promise except for cv. Victor (Table 1). The most productive cultivars in the number of regenerated plants per one hundred zygotic embryos were: Golden Promise, Victor, Atribut, Forum, Amulet. The lowest regeneration capacity was observed in Olbram, Ditta and Primus.

Both quantitative and qualitative parameters of the callus formation seem to depend on the genotype and auxin type. Similar results have been obtained in the model cultivar Golden Promise, a commercial cultivar Galena (Cho *et al*. 1998) other 32 barley cultivars grown in Spain (Castillo *et al*. 1998) and 8 barley cultivars in Poland (Przetakiewicz *et al.* 2003). Callus quality, growth and regenerative potency are affected by a particular type of auxin and cytokinin chosen (Jiang *et al.* 1998). In most of the barley cultivars tested the highest frequency and quality of calluses were achieved on a culture medium supplemented with dicamba. A significant difference among genotypes in somatic embryogenesis and subsequent production of green plants was also indicated. Similar findings were reported by Castillo *et al*. (1998), Barro *et al*. (1999), Przetakiewicz et al. (2003).

Some reports evaluated and screened the tissue culture response of German (Lührs and Lörz 1987), Canadian (Baillie *et al*. 1993), Spanish (Castillo *et al*. 1998), North American (Cho *et al*. 1998) and Polish (Przetakiewicz *et al*. 2003) barley cultivars. The results showed that the tissue culture conditions *in vitro* facilitated somatic embryogenesis and plant regeneration in barley cultivars. Seven lines out of 31 spring-type German barley lines regenerated plants (Lührs and Lörz 1987). The plant regeneration potency also differed among 10 Canadian barley cultivars, ranging from 5 to 41 plants per 100 cultivated zygotic embryos (Baillie *et al*. 1993). The best two-row type cultivars grown in Spain were Reinette with 64 % and Mogador with 49 % plant regeneration frequency (Castillo *et al*. 1998). Barro *et al*. (1999) reported a very high regeneration capacity (59 - 88 %) in three current European spring barley cultivars. Mean number of regenerated plantlets per one embryo in Polish experiments range from 2 for cv. Krona to 58 for cv. Scarlett (Przetakiewicz *et al*. 2003).

In our study, regenerated plants were obtained from all the 24 cultivars tested. These results demonstrated a comparable level of regenerated potency of tested cultivars. We did not observe any relationship between total callus induction and frequency of plant regeneration (r = 0.07). However, the increased number of compact calluses on the media with dicamba achieved in our experiment correlated with a higher number of regenerated plants. The absence of a correlation between the induction frequency of somatic embryos or calli and that of plant regeneration is in agreement with other reports. Komatsuda *et al.* (1989) demonstrated that the rate of callus induction and regeneration ability in barley were two independent processes controlled by independent genetic systems.

In conclusion, it may be said that this study has clearly demonstrated that the induction of somatic embryogenesis and plant regeneration in commercially cultivated barley cultivars is possible with regard to both the qualitative and quantitative aspects which is a pre-requisite for the use of these cultivars for transformation *via* particle bombardment.

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