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Factors affecting the regeneration capacity of isolated barley microspores (*Hordeum vulgare* L.)

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Abstract The culture of isolated microspores of barley (*Hordeum vulgare* L. cv. Kymppi, an elite malting barley cultivar) was studied. A careful choice of culture steps resulted in an average regeneration frequency of 300 green plants per starting material spike. Strong seasonal variation in regeneration capacity was observed. The choice of a cold pretreatment method affected the viability of microspores. A cold pretreatment of the collected starting material at +4°C for 4 weeks was needed for the efficient regeneration of green plants from isolated microspore cultures. Glutamine omission from and copper additions to microspore culture were studied. The omission of glutamine did not affect the number of regenerated green plants but did result in an increase in the number of regenerated albino plants. The addition of copper did not improve the regeneration capacity of isolated barley microspores. Transformation by particle bombardment of isolated microspores did not result in the production of transgenic plants.

Keywords Barley · *Hordeum vulgare* L. · Microspores · Regeneration capacity · Seasonal variation

Introduction

Cereal anther and microspore cultures are widely used in plant breeding programs for the production of doubled haploid lines. Uninucleate haploid microspores are also ideal for gene transfer experiments, with homozygous transgenic plants appearing in the first generation. The culture and regeneration of barley microspores is well established (e.g. Hoekstra et al. 1993; Mordhost and Lörz 1993; Salmenkallio-Marttila and Kauppinen 1995a; Davies and Morton 1998). Transgenic plants have been produced by particle bombardment of isolated microspores (Jähne et al. 1994) and the electroporation of microspore culture-derived protoplasts (Salmenkallio-Marttila et al. 1995b). However, in order to be able to produce high numbers of transgenic plants from isolated uninucleate microspores the isolation and regeneration protocol must be extremely efficient.

The aim of the study reported here was to develop an improved protocol for the regeneration of green plants from isolated barley microspores. The effects of season, pretreatment parameters of starting material, isolation procedures, culture systems and the use of glutamine and copper in culture medium on the regeneration capacity of isolated microspores were evaluated. The ultimate aim was to utilize isolated barley microspores using an improved regeneration protocol as targets for gene transfer experiments by particle bombardment.

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Materials and methods

Plant material

Barley grains (*Hordeum vulgare* L. cv. Kymppi) were potted in a soil mix (Vermiculite: peat: soil; 2:1:1) in a growth chamber [22°/13°C (day/night); 19 h of light at 300 µmol m^{-2} s^{-1}; 40–50% relative humidity] for microspore production. The plants were grown 6–8 weeks and fertilized weekly (Kukkien Y-lannos, Maatalouspalvelu Oy, Finland; Biolan S.M.3, Biolan Oy, Finland; Puutarhan täyslannos, Kemira Oy, Finland) according to the manufacturers' instructions. The spikes were harvested when the sheath of the flag leaf had emerged 5–10 cm, which correlates with the late uninucleate to early binucleate stage of microspore development. Different cold pretreatments of harvested spikes were tested: (A) the collected spikes covered by the flag leaf sheath were wrapped in aluminum foil and kept in water in the dark at +4°C; or the cover of the flag leaf sheath was removed and the spikes were placed (B) in petri dishes with a water insert or (C) on water-moistened filter paper in petri dishes. In the latter two treatments the petri dishes were sealed with Parafilm and kept in the dark at +4°C. The length of the cold pretreatment was also tested (0–4 weeks).

Isolation of microspores

Two isolation methods were adopted: the method of Salmenkallio-Marttila et al. (1995b) utilizes Teflon rod maceration and that of Mordhorst and Lörz (1993), a Waring Micro Blendor blending of spikes. In both methods 0.3 *M* mannitol is used as isolation medium, and the crude microspore preparation is filtered through an 80-µm sieve. Microspores were collected by centrifugation for 10 min at 85 *g*. Different maltose gradients [18%, 19%, 20% and 21% (w/v)] were used for collecting the viable microspores. The viability was determined by staining with fluorescein diacetate (Widholm 1972).

Culture of microspores

For the regeneration capacity studies the culture procedure of Salmenkallio-Marttila et al. (1995b) was modified. Isolated microspores were cultured in 108C medium, which is N6 basal liquid medium (Chu et al. 1975) supplemented with 1.1 m*M* glutamine, 2.8 m*M* myo-inositol, 0.325 *M* maltose (177 g/l, 400 mosmol/kg), 7.9 µ*M* 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.2 µ*M* kinetin, pH 5.8. The omission of glutamine and the addition of copper (1–10 µ*M*) were studied. The purified microspore fraction was resuspended in 108C medium at a density of 0.5×10^5 viable microspores per milliliter in 2.5- or 5-cm (diameter) petri dishes (volumes of 2 and 4 ml, respectively). The cultures were incubated stationary for 1 week and in a rotary shaker for a subsequent 2 weeks (60 rpm; stroke radius, 2.5 cm) in the dark at 22°C.

For comparison, isolated microspores were grown on solid 108C medium (3 g/l Phytagel, Sigma). The purified microspore fraction was resuspended in 108C medium at a density of 3×10^5 viable microspores per 100 µl of 108C medium. A droplet containing approximately 3×10^5 viable microspores was plated on a solid medium, and the liquid was allowed to absorb before the plate was sealed. Cultures were grown in the dark at 22°C for 3 weeks. The addition of 1–10 µ*M* copper to the medium was studied.

Regeneration of plants

The regeneration procedure of Salmenkallio-Marttila et al. (1995b) was adopted. After 3 weeks of culture in the dark at 22°C, calli that had developed were transferred to modified MS (Murashige and Skoog 1962) medium I and cultured further at 22°C in the dark. After 2 weeks, the mass was plated on regeneration medium II and grown in a growth room [24°/13°C (day/night), 16 h of light at 200 µmol m^{-2} s^{-1} supplied by Osram cool-white/Osram fluora (1:1 on watt basis)]. In the regeneration capacity studies, plantlets were counted at 2-week intervals for 4 weeks. For the fertility estimations, plantlets were transferred to medium III for further growth and potted in a soil mix when properly rooted. The regeneration media I, II and III were modified MS-media used by Olsen (1987) solidified with Phytagel (3 g/l). Media I and II contained maltose (60 and 35 g/l, respectively; Hunter 1987), and medium I was also supplemented with 4.5 µ*M* 2,4-D. The addition of copper to the media was studied (1–10 µ*M*; the same addition as to the 108C medium). In the study of glutamine omission, glutamine was only omitted in the initial 108C culture step.

Statistical analyses

The data were processed with the *t*-test or Tukey HSD method of the StatsGraphics program package (version 3.1, Statistical Graphics Corp.).

Results and discussion

The regeneration capacity of isolated barley microspores (*Hordeum vulgare* L. cv. Kymppi) was followed for 1 year. In general, regeneration varied from batch to batch. Strong seasonal variation was observed (Fig. 1). Eventhough the plant material was produced in an artificial growth chamber, it appeared to react according to the season. No explanation for this could be found. The number of regenerated plants was significantly lower if barley grains were potted in soil during the winter (from November to February), whereas better results were obtained with material grown during the spring, summer and autumn. Thus, attempts at transformation should apparently be carried out with plant material grown from March to October. A similar observation with respect to this seasonal variation in regeneration frequency was also reported by Jähne et al. (1994, and personal communication).

Microspores need a signal to be switched from the gametophytic to the sporophytic development pathway.

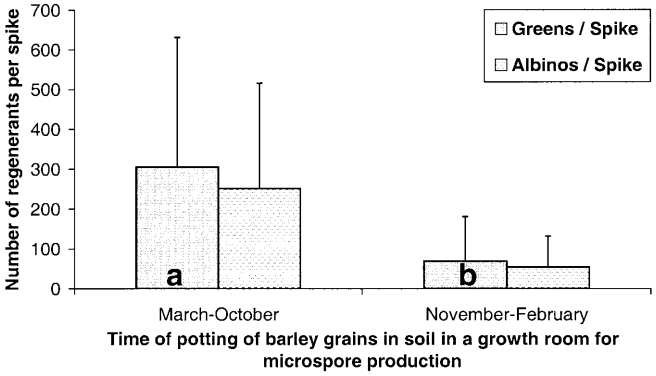

Fig. 1 Strong seasonal variation in the regeneration capacity of isolated microspores (*Hordeum vulgare* L. cv. Kymppi) was observed. The regeneration capacity was followed for 1 year. The number of regenerated green plants marked with *different letters* (*a*, *b*) denotes a statistically significant difference ($P<0.001$) at the 95% confidence level

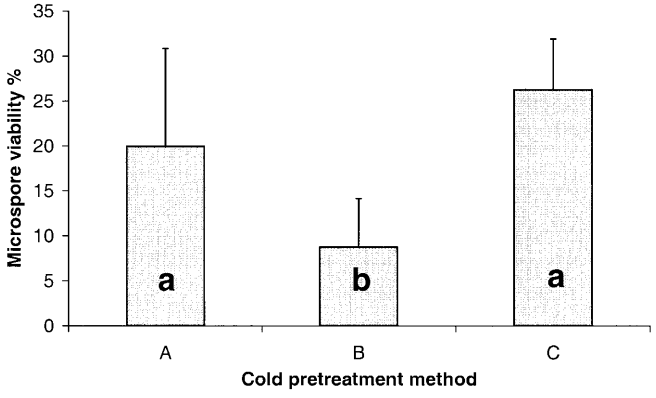

Fig. 2 The cold pretreatment of collected spikes of *H. vulgare* L. cv. Kymppi. *A* The spikes were covered by the flag leaf sheath and wrapped in aluminum foil. *B* and *C* The flag leaf sheath was removed and the spikes were placed in petri dishes with water inserts (*B*) and on water-moistened filter paper in petri dishes (*C*). The viability of a crude microspore sample was then analyzed. *Bars* marked with *different letters* (*a*, *b*) denote a statistically significant difference in viability at the 95% confidence level

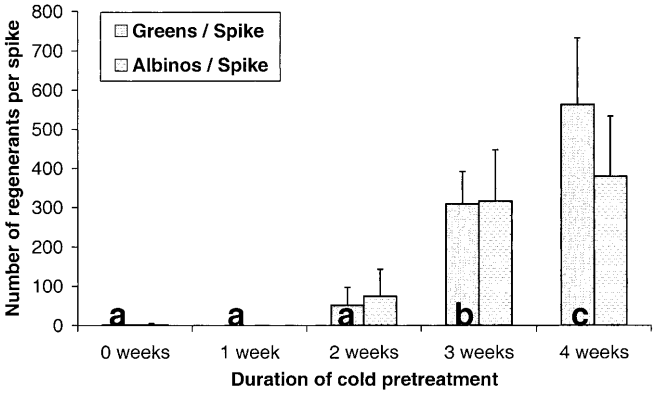

Fig. 3 The effect of a cold pretreatment period on the regeneration capacity of isolated microspores (*H. vulgare* L. cv. Kymppi). The number of regenerated green plants marked with *different letters* (*a, b, c*) denotes a statistically significant difference at the 95% confidence level

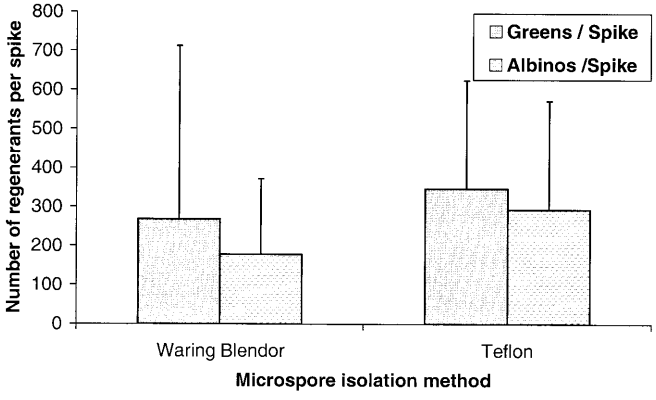

Fig. 4 The comparison of two microspore isolation methods: Waring Blendor blending and Teflon rod maceration. The number of regenerated albino plants marked with *different letters* (*a, b*) denotes a statistically significant difference ($P<0.05$) at the 95% confidence level

Cold shock and osmotic stress pretreatments have been applied to a variety of plant species (for a review see Jähne and Lörz 1995). In barley, a common pretreatment has been the application of cold (+4°C) for 4 weeks in the dark (Mejza et al. 1993). The duration of the cold pretreatment period has also been shown to be genotype-dependent (Powell 1988). The viability of the crude microspore sample after isolation was analyzed and methods A and C (for details see Methods) proved to be equally valid (Fig. 2), with viability averages of 20% and 26%, respectively. The optimal length of the cold pretreatment proved to be 4 weeks at +4°C in the dark (Fig. 3). The regeneration capacity decreased dramatically after 4 weeks (data not shown).

Two microspore isolation methods were compared. The number of green plants regenerated (Fig. 4) after isolation by the Teflon rod maceration method (Salmenkallio-Marttila et al. 1995b) was slightly higher when compared to isolation by the Waring blending of spikes (method of Mordhorst and Lörz 1993). However, the difference was not statistically significant. The number of regenerated albinos was significantly ($P<0.05$) lower at the 95% confidence level if a Waring Blendor was used in isolation. While there is no obvious reason for this observation – Waring micro blendors are made of stainless steel – it has been speculated that some metal ions important to the regeneration of green plants may be released from the blendors. It has been reported that albino plants from anther culture generally carry deletions in their chloroplast genomes (Day and Ellis 1984, 1985). Caredda and co-workers (2000) found that degradation of microspore plastid DNA during early pollen development prevented the plastids from differentiating into chloroplasts under microspore culture conditions. Furthermore, anther apoptosis was strongly cultivar dependent and occurred so early in the development program that the cultivar remained unable to produce green plants regardless of the pretreatments and culture conditions used. It has also been shown that nuclear genes control the formation of albinos from anther cultures (Larsen et al. 1991). The mechanism by which nuclear genes affect the formation of changes in the chloroplast genomes is not known, but changes appear to be induced by factors involved in the cell tissue culture system. However, as has been shown, there are several possible reasons for albinism (Caredda et al. 2000; Hofinger et al. 2000; Torp et al. 2000).

The concentration of maltose in the purification did not have a statistically significant effect (95% confidence level) on the viability of the purified fraction or on the intake of viable microspores. The viability of the purified fraction assessed using fluorescein diacetate was 73±15%, and the intake from viable microspores was 85±11%. Purification with a 20% maltose gradient was utilized in further experiments.

Uninucleate haploid microspores are ideal material for gene transfer, with homozygous transgenic plants already appearing in the first generation. For particle bombardment gene transfer experiments, isolated microspores must be plated on solid culture medium. The use of a solid culture system immediately after isolation did not have a statistically significant effect (95% confidence level) on the regeneration capacity of the isolated microspores (Fig. 5). The bombardment of isolated barley microspores (cv. Kymppi) did not result in transgenic plants. However, as we have shown previously, cv. Kymppi is very recalcitrant to transformation (Nuutila et al. 1999). The reason for this is not known, but it cannot be the regeneration capacity, as demonstrated here. The only report of successful transformation of barley by microspore bombardment is by Jähne et al. (1994). Barley cv. Golden Promise has been shown by several research groups to be transformable by several methods (Wan and Lemaux 1994; Tingay et al. 1997; Nuutila et al. 1999; Zhang et al. 1999). The successful use of Golden Promise microspores as targets for gene transfer remains to be demonstrated.

Jähne and co-workers (1994) reported that the omission of glutamine resulted in a significantly higher re-

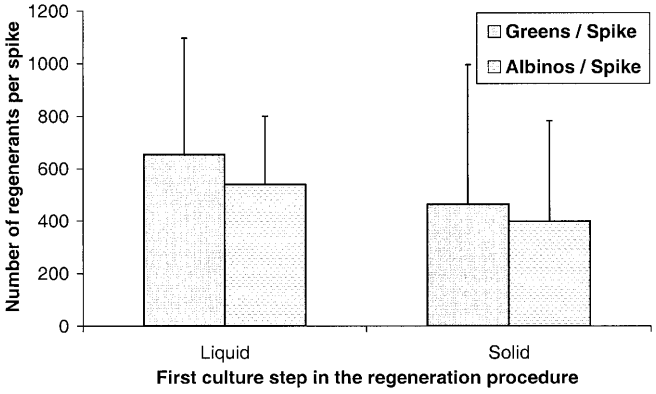

Fig. 5 The effects of liquid and solid first culture steps on the regeneration capacity of isolated barley microspores (*H. vulgare* L. cv. Kymppi). The regeneration capacity did not differ statistically ($P>0.1$, at the 95% confidence level)

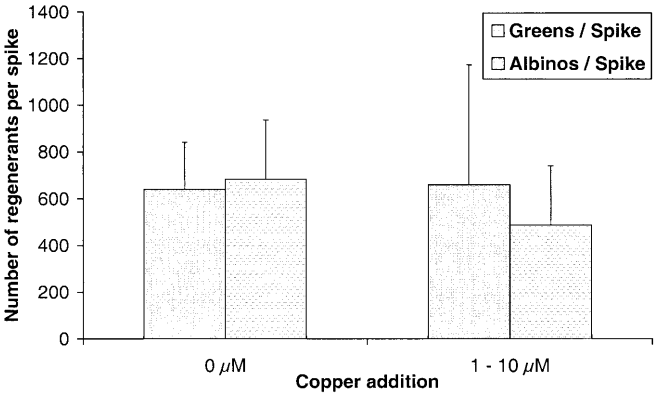

Fig. 7 The effect of copper on the regeneration capacity of isolated barley microspores (*H. vulgare* L. cv. Kymppi). The number of regenerated green plants did not differ statistically ($P>0.1$, at the 95% confidence level)

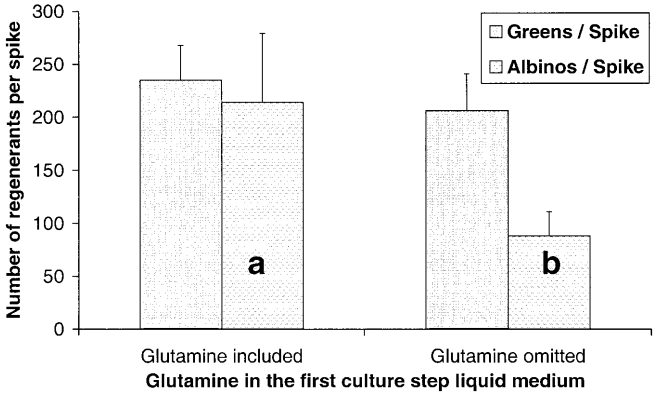

Fig. 6 The effect of glutamine on the regeneration capacity of isolated barley microspores (*H. vulgare* L. cv. Kymppi). The number of regenerated green plants did not differ ($P>0.1$, at the 95% confidence level), but the number of regenerated albino plants marked with *different letters* (*a, b*) denotes a statistically significant difference ($P<0.05$) at the 95% confidence level

generation capacity with barley cv. Igri. Conversely, Olsen (1991), Hoekstra and co-workers (1992) and Mordhorst and Lörz (1993) had reported earlier that glutamine is essential for the culture and regeneration of barley microspores. In our study the omission of glutamine from the first culture step did not have a statistically significant effect (95% confidence level) on the number of regenerated green plants (Fig. 6). However, the number of regenerated albino plants was significantly lower (95% confidence level) when glutamine was omitted. Thus, the use of glutamine appeared to induce albinism, while the number of regenerated green plants remained the same even when glutamine was omitted. Dahleen (1995) and Nuutila et al. (2000) both reported that elevated levels of copper improved the regeneration of green plants from polyembryogenic cultures of barley. However, in our study the addition of copper did not improve the regeneration capacity of isolated barley microspores (Fig. 7).

The regeneration capacity of isolated barley microspores (*H. vulgare* L. cv. Kymppi) varied over a wide range. However, our careful choice of microspore culture steps resulted in an average regeneration frequency of 300 green plants per starting material spike. In order to evaluate the fertility of the microspore culture-derived plants, 76 plants were transferred to soil mix after rooting in regeneration medium III without any colchicine treatment. Of these plants, 59% were fertile, which is in accordance with the reported spontaneous doubling frequency of barley, varying from 50% to over 90% (Pickering and Devaux 1992; Gudu et al. 1993). Thus, our tissue culture protocol did not appear to cause any reduced fertility, and colchicine treatment would probably have further improved the fertility.

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