# Zinc sulphate improved microspore embryogenesis in barley

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**ABSTRACT**

The effect of $ZnSO_4$ concentration on barley (*Hordeum vulgare* L.) microspore embryogenesis was investigated using cultivars of different androgenetic response. Concentrations from 0 (control) to 600 μM in the stress pre-treatment medium alone or in combination with 30 (control) to 600 μM in the embryo induction medium were assayed in anther culture. Incorporation of $Zn^{2+}$ in the pre-treatment medium itself did not affect microspore embryogenesis. The optimum concentration in the stress pre-treatment and induction media was 180 μM for cultivars (cvs.) Igri and Reinette, and 90 μM for cv. Hop. A significant increase of 30 and 300% in cv. Igri and Reinette, respectively, were produced with 180 μM $ZnSO_4$ in both the number of embryos and green plants. In order to confirm the effect of $Zn^{2+}$ on microspore embryogenesis this micronutrient was incorporated in the induction medium of isolated microspore cultures of cv. Igri. Concentrations of 90–300 μM $ZnSO_4$ resulted in an increase of 40–53% in the number of embryos and green plants. All these results indicate that the beneficial effect of $Zn^{2+}$ is exerted mainly during the culture phase, increasing the number of embryos, leading to an increased number of green plants, but it had no effect on percentage of regeneration or green plants.

**Keywords** Doubled haploid - Cereals - Androgenesis - $ZnSO_4$

## INTRODUCTION

The production of doubled haploid (DH) is a highly valuable tool for plant breeding, since completely homozygous lines from $F_1$ crosses are obtained in a single generation. Barley has been considered as a model species for microspore embryogenesis among cereals. Efficient protocols for DH production have been developed for some model cultivars via anther and/or isolated microspore cultures. However, there are some agronomically important cultivars that still render none or a low number of green plants (Muñoz-Amatriaín et al. 2008). The development of efficient protocols for recalcitrant barley genotypes can be very useful for a better understanding of physiological factors playing an important role in this process, as well as for optimization of microspore embryogenesis in other cereals.

Modifications of stress pre-treatment, carbohydrate and nitrogen source, and growth regulators of culture media has led to significant improvements in the number of barley DH plants (Maluszynski et al. 2003). Less attention has been paid to the micronutrient composition of the media. To our knowledge, only the effect of $Cu^{2+}$ has been reported to increase the yield of green plants in the model barley cv. Igri (Wojnarowiez et al. 2002). $Zn^{2+}$ is another important micronutrient which plays a fundamental role in many physiological and biochemical cellular processes, such as structural and functional integrity of biomembranes, photosynthesis, auxin metabolism, response to oxidative stress, apoptosis inhibition, phosphate transport, etc. (Marschner 1995; Cakmak 2000; Huang et al. 2000; Chimienti et al. 2001; Hoseini and Mulligan 2002). Zinc is also an essential component of enzymes and transcription factors involved in plant morphogenesis and organogenesis (Kobayashi et al. 1998; Yanagisawa 2004).

Studies on the influence of this micronutrient in callus, cell suspensions and somatic embryogenesis are very limited. Concentration from 52 to 520 μM in rice and 1,000 μM in tobacco resulted in increased growth (Hirt et al. 1989; Hossain et al. 1997), whereas 10 mM became toxic in tobacco (Bueno and Piqueras 2002). The elimination of $Zn^{2+}$ stimulated somatic embryogenesis in wheat (He et al. 1991). However, a tenfold decrease of its concentration (3 μM) did not affect somatic embryogenesis in chilli pepper (Kintzios et al. 2001). In barley, an increase of $Zn^{2+}$ from 30 to 52 μM resulted in an enhanced callus formation in leaf segments (Pasternak et al. 1999). Requirements of this micronutrient seem to be species dependent and the optimization of its

concentration in the culture media has led to the improvement of different culture systems. To our knowledge, the effect of zinc concentration on microspore embryogenesis has not been studied previously. In this study, we examined the influence of different concentrations of $ZnSO_4$ in the stress pre-treatment and/or in embryo induction media on microspore embryogenesis of barley cultivars with high, medium and low androgenetic response.

## MATERIAL AND METHODS

The winter two-rowed barley cultivars (cvs.) Igri and Reinette, and the winter six-rowed cv. Hop were used for the evaluation of anther culture, and cv. Igri for microspore culture. Igri is a model cultivar for microspore embryogenesis, whereas Reinette and Hop are medium and low responding cultivars, respectively. Well-established standard protocols described by Cistué et al. (2003) and Castillo et al. (2000) were followed for evaluation of anther and isolated microspore culture response, respectively.

### Effect of zinc sulphate concentration on anther culture efficiency

Effect of zinc sulphate concentration in the pre-treatment medium

The three anthers dissected from each of the six central flowers and both sides of three spikes (from different plants), were randomly distributed in 0.7 M mannitol, 40 mM $CaCl_2$ solidified with 8 g $l^{-1}$ Agarose Sea Plaque (pre-treatment medium), containing 0 (Control), 30, 90, 180, 300 and 600 μM of $ZnSO_4$ (18 anthers cultured per Petri dish). Anthers were pre-treated for 4 days at 25°C in the dark. Anthers were transferred to 2 ml of FHG liquid induction culture medium (Cistué et al. 2003) supplemented with 200 g $l^{-1}$ of Ficoll Type-400 (Sigma) (FHG-F200) in 3 cm diameter plates. FHG medium (Hunter 1988) is a modified MS (Murashige and Skoog 1962) medium, containing 165 mg $l^{-1}$ $NH_4NO_3$, thiamine HCl 0.4 mg $l^{-1}$ (as vitamin source), glutamine 730 mg $l^{-1}$ and maltose 62 g $l^{-1}$.

Effect of $ZnSO_4$ in the pre-treatment and the culture media of anther culture

In this experiment, the three anthers from each of the five central flowers and both sides of three spikes were dissected and distributed in five pre-treatment media (containing 0, 90, 180, 300 and 600 μM $ZnSO_4$). The 18 anthers from each stress pre-treatment medium were transferred to 2 ml FHG-F200 containing the same concentration of $ZnSO_4$, with the exception of control cultures. Anthers pretreated in 0 μM $ZnSO_4$ and cultured in 30 μM of $ZnSO_4$ (concentration of FHG medium) were used as control. After 12–14 days of culture at 25°C in the dark, the plates were replenished with 2 ml of FHG medium with 400 g $l^{-1}$ Ficoll containing the same amount of $ZnSO_4$ as before.

**Effect of $ZnSO_4$ concentration in the culture medium of isolated microspores**

Microspores were isolated from anthers of cv. Igri pretreated for 4 days on the control pre-treatment medium described above, and were cultured in FHG liquid medium containing 100 g $l^{-1}$ Ficoll and 30 (control), 90, 180 and 300 μM of $ZnSO_4$ at a final density of $10^5$ microspores $ml^{-1}$ in 3 cm diameter plates. Cultures were incubated at 25°C in the dark. Six to nine days after culture, 1 ml of the medium containing the same amount of $ZnSO_4$ as before, and supplemented with 300 g $l^{-1}$ Ficoll was added to the Petri dishes. Six days later, cultures were transferred to 6 cm diameter Petri dishes containing 8 ml of fresh FHG medium with 300 mg $l^{-1}$ Ficoll.

Twenty to forty days after anther or isolated microspore culture, embryos were subcultured on regeneration medium (Castillo et al. 2000; Cistué et al. 2003). Twenty plants from each zinc sulphate concentration were transferred to soil (as described by Cistué et al. *2003*) for further development and data analysis of seed production.

**Data analysis**

Anther culture experiments consisted of 12–20 replicates (18 anthers cultured in the same Petri dish) for each concentration and genotype tested, using two different batches of plants. The following variables were recorded: number of embryos (nEMB), green plants (nGP), albino (nAP), all of them referred to 100 anthers; percentage of regeneration (number of total plants per 100 embryos; pREG); percentage of green plants (number of green plants per total plants; pGP).

Fifteen spikes were used for each of the three microspore isolation experiments using three different batches of plants. The same variables as those for anther culture, plus number of dividing microspores (nDIV) and percentage of embryogenesis (number of embryos/number of dividing microspores, pEMB) were recorded. Variables nDIV, nEMB, nGP, nAP were referred to $10^5$ microspores. The variable nDIV was estimated by counting the number of dividing microspores producing globular embryos, in one-tenth of the Petri dish area on a millimetre paper under a stereoscopic microscope.

Statistical analyses were carried out using standard SAS/STAT procedures. Variables percentage of embryogenesis (pEMB) and nGP were transformed with log (x + 1) for data normalization. Analysis of variance was carried out using the Generalized Linear Model procedure. Means separation was tested by the Duncan method.

## RESULTS

### Effect of $ZnSO_4$ concentration in the pre-treatment medium of anther culture

The number of embryos and green plants increased progressively when $ZnSO_4$ concentration was raised from 30 to 180 μM, in cv. Igri, although this increase was not significant statistically (around a 40% enhancement when compared with control). Higher concentrations produced similar values to control (Table 1). In cv. Igri, the number of albino plants increased significantly, up to almost three times in medium containing 30, 90 and 600 μM as compared with control. However, percentage of green plants did not vary significantly with any of the concentrations used. In both cvs. Reinette and Hop, no significant differences were found for any of the variables. However the number of green plants from cvs, Reinette and Hop in medium containing 90 and 30 μM was doubled as compared with control.

### Effect of $ZnSO_4$ in the pre-treatment and the culture media of anther culture

The number of both embryos and green plants was enhanced significantly when the concentration of $Zn^{2+}$ in the pre-treatment and the culture medium was raised up to 180 μM for cvs. Igri and Reinette (Table 2). Around a 1.3-fold increase for Igri and a threefold increase for Reinette in these two variables were obtained. In Reinette, the

number of albino plants also increased significantly up to three times with 180 μM. No statistically significant differences for number of embryos, green and albino plants were obtained for cv. Hop (probably due to its low response ant its high rate of variation in response, data not shown), although a two to fourfold increase in these variables were produced with 90 μM. No statistically significant differences were obtained either for the percentages of green plants, or the percentage of regeneration in any of the three cultivars. $Zn^{2+}$ does not seem to affect the quality of the embryos hence the percentage of regeneration did not change with high concentrations.

**Effect of $ZnSO_4$ concentration in the culture medium of isolated microspores**

To confirm and support the results obtained in anther culture, different concentrations of this micronutrient were assayed in microspore culture medium of cv. Igri. The number of embryos and green plants were raised significantly when the concentration of $ZnSO_4$ was increased from 30 to 300 μM (Table 3). Up to 269 embryos/$10^5$ microspores and 253 green plants/$10^5$ microspores were obtained with 90 μM, which represents an increase of the 43 and 37% in the number of embryos and green plants, respectively, as compared with control. Percentage of embryogenesis was also raised significantly from 6.65 in control up to 9.57 with 90 μM. Similar values in these three variables were obtained in a wide concentration range, from 90 to 300 μM. The concentration of this micronutrient did not affect the number of dividing microspores, percentage of green plants and percentage of regeneration.

There were no significant differences in morphological characters among the plants produced from control cultures and 90 to 600 μM of $ZnSO_4$ in the culture medium (data not shown).

## DISCUSSION

Zinc is an essential micronutrient in plants and thus is included in all plant tissue culture media, normally supplied as $ZnSO_4$ in amounts of 3–30 μM. Although studies about the influence of this micronutrient are limited, requirements of $Zn^{2+}$ seem to be species- and system-dependent (He et al. 1991; Hossain et al. 1997; Teasdale 1986). This is the first study about the influence of $ZnSO_4$ on microspore embryogenesis. This study shows

that 90–180 μM in the stress pre-treatment and induction medium increased significantly the number of embryos and green plants, without affecting the percentage of green plants and regeneration, in both anther and isolated microspore cultures. A lower concentration of $ZnSO_4$ seems to be more beneficial for the low androgenetic responding cv. Hop compared to the medium and high responding cvs. Reinette and Igri, respectively. These results could indicate a higher sensitivity of cv. Hop to this micronutrient. A high concentration of this micronutrient could be toxic as described in suspension cells of tobacco with 10 mM (Bueno and Piqueras 2002). However, no toxic effect was observed in this study with the highest concentration used.

The benefit of $Zn^{2+}$ is exerted mainly during the initial culture phase, since increased concentration of this micronutrient in the culture medium did significantly raise the number of embryos and green plants in isolated microspore cultures. A lower concentration of $ZnSO_4$ in isolated microspore culture compared to anther culture seems to exert a similar beneficial effect on microspore embryogenesis in cv. Igri. This could be due to the fact that the anther wall could interfere with the absorption of this micronutrient by the microspores. Pulido et al. (2005) already described that anther tissues could act as a filter to prevent high concentrations of Fe from reaching the microspores.

$Zn^{2+}$ seems to play an important role in the biosynthesis and metabolism of auxin (Skoog 1940; Tsui 1948; Cakmak et al. 1989). Processes regulated by auxins were stimulated with a high concentration of this micronutrient (Hossain et al. 1997; Saeki et al. 2000; Oguchi et al. 2004a, b). $Zn^{2+}$ modulated auxin action by regulation of different proteins including an ascorbato peroxidase and a late embryogenesis abundant (LEA) protein (Oguchi et al. 2004a, b). Functional genomic analysis of barley microspores after 4 and 8 days of culture showed up-regulation of auxin-related proteins, LEA proteins and ascorbate oxidase promoter-like binding protein (M.P. Vallés, unpublished data). This micronutrient is also an essential component of zinc finger transcription factors involved in plant morphogenesis and organogenesis (Kobayashi et al. 1998; Yanagisawa 2004). $Zn^{2+}$ could also play an important function in the reprogramming of the microspores to the sporophytic pathway. The expression of several zinc finger proteins has been described to play an important role during zygotic

embryogenesis in Arabidopsis (Li and Thomas 1998; Xu and Li 2003) and in animal embryogenesis (Falchuk and Montorzi 2001).

In this study we have increased the microspore embryogenesis capacity of barley cultivars with different androgenetic response, by raising $ZnSO_4$ concentration up to 90–180 μM in the stress pre-treatment and culture media in anther culture and up to 90–300 μM in induction medium of isolated microspore cultures. Molecular and biochemical approaches should be used for further understanding the role of zinc in the stimulation of microspore embryogenesis.

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**Table 1** Effect of zinc sulphate concentration in the pre-treatment medium on anther culture of cultivars Igri, Reinette and Hop

| Genot | Zinc Con (µM) | Nant | EMB per 100 Anthers | GP per 100 Anthers | AP per 100 Anthers | pGP | pREG |
|---|---|---|---|---|---|---|---|
| Igri | Control (0) | 234 | 171.7 a* | 124.8 a | 8.5 b | 93.6 a | 77.6 a |
| | 30 | 270 | 222.2 a | 150.7 a | 23.3 a | 86.6 a | 78.3 a |
| | 90 | 270 | 263.0 a | 168.1 a | 23.3 a | 92.7 a | 69.0 a |
| | 180 | 270 | 250.4 a | 178.1 a | 14.4 ab | 92.5 a | 76.9 a |
| | 300 | 270 | 204.1 a | 126.3 a | 16.3 a | 88.6 a | 69.9 a |
| | 600 | 270 | 194.4 a | 130.3 a | 20.0 a | 86.7 a | 77.3 a |
| Reinette | Control (0) | 108 | 65.0 a | 11.7 a | 28.1 a | 29.4 a | 61.3 a |
| | 30 | 108 | 81.9 a | 12.3 a | 31.6 a | 28.1 a | 53.6 a |
| | 90 | 108 | 75.1 a | 27.4 a | 21.5 a | 56.0 a | 65.1 a |
| | 180 | 108 | 84.3 a | 21.5 a | 17.9 a | 54.7 a | 46.7 a |
| | 300 | 108 | 62.4 a | 13.8 a | 20.8 a | 40.0 a | 55.4 a |
| | 600 | 108 | 55.9 a | 8.3 a | 15.0 a | 35.7 a | 41.7 a |
| Hop | Control (0) | 162 | 31.5 a | 7.4 a | 12.3 a | 39.1 a | 45.0 a |
| | 30 | 162 | 49.4 a | 14.8 a | 18.5 a | 27.4 a | 61.4 a |
| | 90 | 162 | 36.4 a | 6.8 a | 11.7 a | 44.6 a | 66.4 a |
| | 180 | 162 | 32.7 a | 4.3 a | 14.8 a | 50.0 a | 28.9 a |
| | 300 | 162 | 29.6 a | 6.2 a | 8.0 a | 54.8 a | 45.6 a |
| | 600 | 162 | 41.3 a | 9.8 a | 16.0 a | 26.3 a | 27.5 a |

* Variable within each genotype followed by the same letter is not statistically different according to a Duncan test ($P \leq 0.05$)

**Table 2** Effect of zinc sulphate concentration in the pre-treatment and culture media on anther culture of cultivars Igri, Reinette and Hop

| Genot | Zinc Con (µM) | | N Ant | EMB | GP | AP | pGP | pREG |
|---|---|---|---|---|---|---|---|---|
| | Pret | Ind | | per 100 Anthers | | | | |
| Igri | Control | 30 | 270 | 299.6 b* | 248.5 b | 16.3 a | 94.1 a | 88.2 a |
| | 90 | 90 | 270 | 283.7 b | 217.7 bc | 11.5 a | 94.9 a | 80.8 a |
| | 180 | 180 | 270 | 406.3 a | 314.8 a | 18.1 a | 95.0 a | 81.9 a |
| | 300 | 300 | 270 | 368.8 ab | 286.3 ab | 20.0 a | 93.5 a | 83.0 a |
| | 600 | 600 | 270 | 291.5 b | 218.5 bc | 17.8 a | 92.5 a | 81.1 a |
| Reinette | Control | 30 | 198 | 68.7 b | 15.1 b | 20.2 b | 42.8 ab | 51.4 a |
| | 90 | 90 | 198 | 119.7 ab | 43.9 a | 31.3 b | 58.3 a | 62.8 a |
| | 180 | 180 | 198 | 229.8 a | 42.4 a | 60.1 a | 41.0 ab | 44.6 a |
| | 300 | 300 | 198 | 126.8 ab | 26.8 ab | 42.9 ab | 38.4 ab | 55.0 a |
| | 600 | 600 | 198 | 75.7 b | 8.6 b | 26.3 b | 24.6 b | 46.1 a |
| Hop | Control | 30 | 216 | 5.1 a | 0.9 a | 1.4 a | 39.1 a | 45.0 a |
| | 90 | 90 | 216 | 12.5 a | 3.7 a | 4.6 a | 44.6 a | 66.4 a |
| | 180 | 180 | 216 | 9.7 a | 1.4 a | 1.4 a | 50.0 a | 28.9 a |
| | 300 | 300 | 216 | 9.2 a | 2.3 a | 1.9 a | 54.8 a | 45.6 a |
| | 600 | 600 | 216 | 6.9 a | 0.5 a | 1.4 a | 26.3 a | 27.5 a |

* Variable within each genotype followed by the same letter is not statistically different according to a Duncan test ($P \leq 0.05$)

**Table 3** Effect of zinc sulphate concentration in the induction medium of isolated microspore cultures of cv. Igri (data are referred to $10^5$ microspores)

| Zinc Con (μM) | nDIV | nEMB | nGP | | | |
|---|---|---|---|---|---|---|
| | per $10^5$ MIC | | | pGP | pEMB | pREG |
| Control (30 μM) | 2825 b* | 188 b | 184 b | 99.2 a | 6.65 b | 97.6 a |
| 90 μM | 2811 b | 269 a | 253 a | 99.8 a | 9.57 a | 94.1 a |
| 180 μM | 2926 b | 256 a | 240 a | 99.6 a | 8.75 a | 93.8 a |
| 300 μM | 3217 ab | 291 a | 257 a | 99.6 a | 9.05 a | 88.2 ab |

* Variable followed by the same letter is not statistically different according to a Duncan test ($P < 0.05$)