

## The Effect of Copper on Plant Regeneration in Barley Microspore Culture

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### Abstract

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Isolated microspore culture is an excellent system for the production of doubled haploids in many crops, including barley. In a more traditional barley anther culture method copper sulphate is known to enhance plant regeneration. Here we report that one hundred times higher concentration of copper sulphate in the isolated microspore culture of two spring barley genotypes compared to the standard content in the induction medium resulted in a 34% increase of total plant regeneration. Detailed analysis of plant regeneration showed that additional supplementation of copper sulphate increased not only the regeneration of green plants but also proportionately that of albino plants. Hence, the results from two studied genotypes do not support an assumption that the addition of copper reduces albinism in barley microspore culture.

**Keywords:** albinism; androgenesis; doubled haploid; *Hordeum vulgare*; regeneration efficiency

Plant homozygosity is desirable in breeding, where stability in successive generations not only improves the resolution of replicated trials but also is a requirement for cultivar registration. In conventional plant breeding homozygous lines are obtained by repeated self-fertilizations, usually for six-seven-eight generations. The techniques used in biotechnology based on *in vitro* culture reduce the time required to achieve homozygosity to a single generation (FORSTER *et al.* 2007). In cereals, the techniques based on haploid embryogenesis are employed the most frequently (DEVAUX & KASHA 2009). Androgenesis is a process of embryos development without fertilisation, from microspores, following a treatment with exogenous stress. As a result, after spontaneous or induced diploidization, completely homozygous plants – doubled haploids (DH) are obtained (KASHA *et al.* 2001). Androgenesis may be performed using two approaches: anther culture (AC) which is labour-intensive but applied more extensively, and a more technologically advanced method by isolated microspore culture (IMC). In the IMC large numbers e.g.  $6 \times 10^6$  of hap-

loid cells capable of synchronous development can be cultured in a small volume (1 ml) of medium (JÄHNE & LÖRZ 1995; OLESZCZUK *et al.* 2006). The relevant literature lacks a definite answer which of these two methods yields better results not only in barley (LI & DEVAUX 2003; LANTOS *et al.* 2014). In some reports, the number of green plants (GP) obtained via IMC was up to nine times higher than in AC (LI & DEVAUX 2005). These differences were explained by better availability of the induction medium nutrients to microspores whereby in IMC cells divide efficiently, develop into the embryos directly and better converse into plants. However, mortality of microspores in IMC is much higher than in AC, which results in the lower number of regenerated plants (CASTILLO *et al.* 2000).

High efficiency of androgenesis, measured by the number of GP regenerated from microspore-derived androgenic structures (AS), is the prerequisite for practical use in research and breeding purposes. This efficiency is dependent on numerous elements (GERMANÁ 2011); the most important among them is the genotype of donor plants. Nevertheless, the

efficiency of DH line regeneration may be regulated to a certain extent by selecting external factors such as: composition of induction and regeneration media, and the choice of the *in vitro* culture method (OLESZCZUK *et al.* 2006; GERMANÁ 2011).

One of the impediments to DH production via androgenesis is the albino plant (AP) regeneration. Such plants are useless and they decrease the efficiency of the entire process (MAKOWSKA *et al.* 2015). The frequency of AP in androgenesis may be up to 100% with the genotype being the strongest determinant of this phenomenon (LARSEN *et al.* 1991; CAREDDA *et al.* 2000). The causes of albinism resulting from androgenesis are still unclear. Studies in the field have identified QTLs in nuclear genome associated with AP production, plastid genome rearrangements identified for plants without chlorophyll and cytological features characterised for plastid inability to transform into chloroplasts (HOFINGER *et al.* 2000; CAREDDA *et al.* 2004; MUÑOZ-AMATRIAIN *et al.* 2008, reviewed in MAKOWSKA & OLESZCZUK 2014). The frequency of AP can be reduced by using appropriate external factors, one of them being copper ions supplemented to the pretreatment solution and induction medium (NUUTILA *et al.* 2000; WOJNAROWIEZ *et al.* 2002).  $\text{Cu}^{2+}$  ions are among heavy metals which, in high concentrations, are toxic and mutagenic for plants. On the other hand, this element is necessary for growth and development of plants. Around 50% of  $\text{Cu}^{2+}$  contained in a cell is located in chloroplasts where it is directly engaged in electron transport during photosynthesis and lack of them is evidenced by chlorosis (MAKSYMIEC 1998).  $\text{Cu}^{2+}$  in the form of hydrated copper sulphate is commonly used as a microelement in various culture media (MURASHIGE & SKOOG 1962; MALUSZYNSKI *et al.* 2003). Increasing the  $\text{Cu}^{2+}$  concentration by almost 100 times in induction media and adding it to the pretreatment solution improved the androgenic response, plant regeneration, as well as the ratio of green to albino plants (WOJNAROWIEZ *et al.* 2002; JACQUARD *et al.* 2009), even though the earlier report did not show such a dependence (RITALA *et al.* 2001). It seems essential to confirm the effect of the  $\text{CuSO}_4$  compound for plant regeneration efficiency and albinism reduction in a different experimental system, which is the IMC.

## MATERIAL AND METHODS

**Plant material and growing conditions.** Two genotypes of spring barley (NAD2 and NAD19) were kindly supplied by Poznan Plant Breeders, Ltd. The

selected lines are characterised by high androgenic yield and high proportions of albino plants regenerated. Donor plants were grown in 18-cm pots with a soil-sand mixture (3:1) in a growth chamber at 16/12°C day/night temperature, under 16-h days with light intensity of approximately 240  $\mu\text{E}/\text{m}^2/\text{s}$  provided by sodium lamps until the appropriate stage of development.

**Pretreatment of donor spikes.** The developmental stage of microspores was checked under a microscope in anthers from flowers located in the middle of the spikes. Tillers were collected when the majority of microspores were at the mid-uninucleate stage. Spikes were removed from tillers and surface-sterilized in 70% ethanol for 1 min followed by 10% sodium hypochlorite for 20 min, and rinsed five times with sterile water. Five spikes from the same genotype were incubated in a 90-mm Petri dish with filter paper and 5 ml of sterile distilled water to maintain high humidity. These spikes were pretreated at 4°C in the dark for 21 days.

**Isolation and culture of microspores.** Microspores were isolated and purified following the procedures of CORONADO *et al.* (2005). The initial densities of microspores in the induction medium were  $1.5 \times 10^5$  per ml. Microspores were suspended in two types of media: KBP (KUMLEHN *et al.* 2006) as a control and KBP supplemented with 10  $\mu\text{M}$   $\text{CuSO}_4 \times 5 \text{H}_2\text{O}$  solution as the experimental variant. One ml of aliquots was cultured in a 35-mm Petri dish in the dark at 27°C. After 2 weeks of culture the first androgenic structures were observed. At this point about one-half of the medium volume was removed and replaced by the same volume of fresh medium. The AS about 1.5 mm in diameter were transferred onto K4ND regeneration medium (KUMLEHN *et al.* 2006); at this point the numbers of AS were assessed and then incubated at 27°C under a 16-h photoperiod regime. The numbers of GP and AP were counted two to three weeks after the AS transfer.

**Microspore viability.** After 7 days of microspore culture a 10  $\mu\text{l}$  sample from every Petri dish was taken to test the viability of cells under a microscope. The viability was assessed based on visual cytologic features: plasmolysis and cell volume. No staining was used. For every sample 10 fields of view were counted for live and dead cells.

**Data analysis.** For each microspore isolation, which was considered as a single experiment, 15 spikes were used. Each isolation produced a different number of cell-culture dishes with a certain microspore density.

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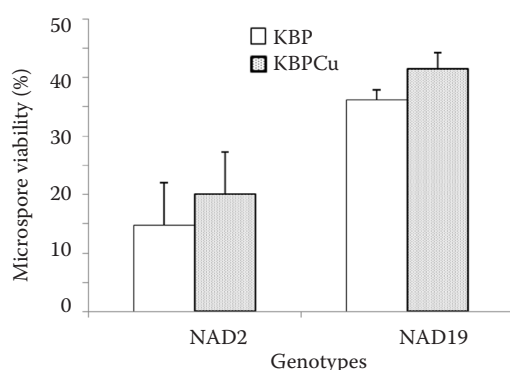


Figure 1. Effect of 10  $\mu\text{M}$   $\text{CuSO}_4$  in induction medium on barley microspore viability after 7 days of culture  
The error bars represent standard error

The dishes were randomly assigned to both treatment groups, e.g. the control group and the group with increased copper sulphate content. In total, four independent experiments were conducted, each with average of 20 dishes. To estimate the percentage of microspore viability more than 22 600 cells were counted for both genotypes. Each number represents the mean ( $n = 4$  for each genotype or  $n = 8$  for both genotypes),  $\pm$  standard error (SE). In figures, error bars represent standard error. The statistical significance was tested using Student's t-test. Differences were considered significant at  $P < 0.05$ .

## RESULTS

**Androgenic response.** The effect of  $\text{Cu}^{2+}$  on isolated microspores was assessed after 7 days of culture. For NAD2, the viability of microspores in the control and in the experimental variant was 15% and 17%, respectively. For NAD19, the viability of cells was 36% in the control and 41% in the experimental group. No significant differences in the viability of microspores resulting from  $\text{CuSO}_4$  supplementation were noticed, however, the differences were significant between genotypes (Figure 1).

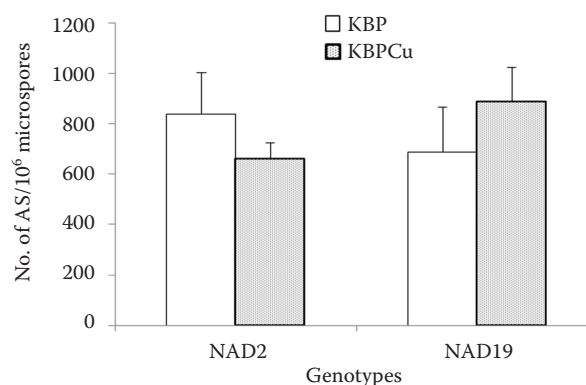


Figure 2. Frequency of androgenic structures formation in barley on different induction media with or without copper sulphate  
The error bars represent standard error; AS – androgenic structure

The number of androgenic structures per  $10^6$  microspores did not differ significantly between analysed conditions in both genotypes. In NAD2, higher concentration of  $\text{CuSO}_4$  in the medium reduced AS formation by ca. 18%. In NAD19, higher concentration of copper sulphate in the induction medium enhanced the production of AS by about 12% (Figure 2).

**Plant regeneration.** Cumulated results for both genotypes tested indicate that  $\text{CuSO}_4$  supplementation significantly increased the total plant (TP) regeneration efficiency by about 34% relative to the control (Table 1). In NAD2 we observed the statistically significant enhancement of TP production as a result of  $\text{CuSO}_4$  supplementation ( $100 \pm 16$  vs.  $74 \pm 20$  TP per  $10^6$  microspores,  $P < 0.05$ ). For NAD19, the increase was of similar magnitude but was not statistically significant ( $90 \pm 25$  vs.  $67 \pm 22$  TP per  $10^6$  microspores, NS) in experimental variant vs. control (Figure 3).

The GP regeneration cumulated for both genotypes was significantly increased by addition of  $\text{CuSO}_4$

Table 1. Parameters of androgenesis and plant regeneration in microspore culture for barley genotypes NAD2 and NAD19

Medium	Microspores				
	No.	AS/ $10^6$	TP/ $10^6$	GP/ $10^6$	AP/ $10^6$
KBP	$6.5 \times 10^6$	$761 \pm 118$	$71 \pm 14$	$14 \pm 3$	$57 \pm 12$
KBP + 10 $\mu\text{M}$ $\text{CuSO}_4$	$6.0 \times 10^6$	$773 \pm 82$	$95 \pm 14^*$	$24 \pm 5^*$	$71 \pm 10^*$

\*statistically significant difference compared to the corresponding control group,  $P < 0.05$ ; each number is a mean ( $n = 8$ )  $\pm$  standard error; number of androgenic structures (AS), total plants (TP), green plants (GP) and albino plants (AP) regenerated per  $10^6$  isolated microspores

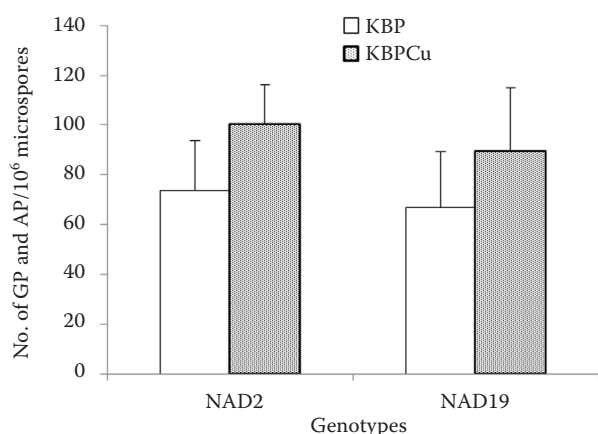


Figure 3. Influence of copper sulphate on total plant regeneration in barley

The error bars represent standard error; GP – green plants; AP – albino plants

(Table 1). The increase was statistically significant for NAD2 ( $26 \pm 3$  vs.  $16 \pm 5$  GP per  $10^6$  microspores,  $P < 0.05$ ), while for NAD19 the increase was of similar magnitude again but was not statistically significant ( $11 \pm 4$  vs.  $23 \pm 10$  GP per  $10^6$  microspores, NS) in experimental and control conditions (Figure 4).

Supplementation of copper sulphate into the induction medium also affected the regeneration of AP. For both genotypes the number of AP increased by about 26% with increased copper concentration relative to the control (Table 1). The result was statistically significant for NAD2 ( $74 \pm 13$  vs.  $58 \pm 15$  AP per  $10^6$  microspores,  $P < 0.05$ ) in the test and control groups, while for NAD19 we observed a similar but not significant results ( $67 \pm 18$  vs.  $56 \pm 20$  AP per  $10^6$

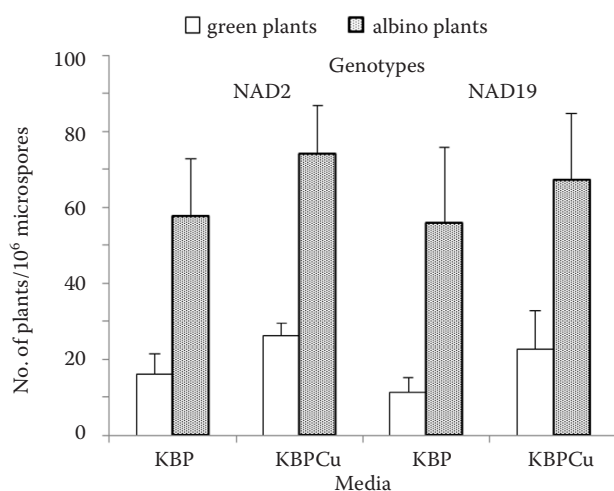


Figure 4. Effect of 10  $\mu\text{M}$   $\text{CuSO}_4$  in induction medium on green and albino plant regeneration in barley

The error bars represent standard error

microspores, NS) (Figure 4). In analysed genotypes the increase of GP regeneration was higher than the AP production. The green to albino ratio for both analysed groups was almost at the same level near 1:3.

## DISCUSSION

The IMC offers, in a short time, a large number of selected cells potentially capable of development into embryos. Compared to AC, this method allows a broader application in basic research such as cytology, embryology and genetic engineering (GONZÁLEZ-MELENDI *et al.* 2005; KUMLEHN *et al.* 2006; DAGHMA *et al.* 2012). The applicability of the isolated microspore system is dependent on the proportion of obtained embryos converted into green plants. For this reason much effort is dedicated to establishing efficient protocols of the microspore culture (ESTEVEZ *et al.* 2014). It is well documented that in barley anther culture additional  $\text{Cu}^{2+}$  supplementation during both the pretreatment and the induction phase of androgenesis improves GP regeneration (JACQUARD *et al.* 2009). The IMC, despite relying on essentially the same process as AC, may react differently to the same compounds added into the media (RITALA *et al.* 2001; WOJNAROWIEZ *et al.* 2002, CASTILLO *et al.* 2014). This is the main reason why it appeared reasonable to verify whether the positive reaction to increased  $\text{Cu}^{2+}$  in the medium observed in AC can also be repeated in IMC. The results demonstrate that the increased  $\text{CuSO}_4$  content in an induction medium does not contribute in a significant way to improving microspore viability after 7 days of culture. This is consistent with the effects presented by WOJNAROWIEZ *et al.* (2002). However, those authors stressed that survivability of haploid cells can be increased by adding  $\text{CuSO}_4$  to the induction medium as well as during the pretreatment. Similarly to JACQUARD *et al.* (2009), we observed a large discrepancy in androgenic reactions to increased  $\text{Cu}^{2+}$  depending on the genotype. This reconfirms the major effect of the genotype of donor plants on the androgenic response. No clear effect of  $\text{Cu}^{2+}$  on the number of AS formed from the microspores was observed.

Positive results were obtained at the plant regeneration step. Enriching the induction medium with a 10  $\mu\text{M}$   $\text{CuSO}_4$  solution already had a beneficial impact on the TP regeneration efficiency for both genotypes. The effectiveness of regeneration measured by TP per  $10^6$  isolated microspores was improved by 34%



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relative to the control group. A similar increase in plant regeneration per 100 anthers was also reported for barley (JACQUARD *et al.* 2009). A distinctly larger improvement in plant regeneration was obtained by WOJNAROWIEZ *et al.* (2002) for cv. Igri, the model cultivar of barley, with an almost 4.5 times increase relative to the control. In both quoted papers, copper sulphate was also added to the pretreatment solution. However, earlier RITALA *et al.* (2001) showed no effect of 10  $\mu\text{M}$   $\text{CuSO}_4$  on the regeneration capacity of barley IMC.

Many authors emphasise that the factor which crucially limits the efficiency of androgenesis is albinism among of regenerated plants. It would seem that, given the cellular functions performed by  $\text{Cu}^{2+}$ , additional supplementation into the medium may have a beneficial impact on reducing this problem (NUUTILA *et al.* 2000; GRAUDA *et al.* 2014). According to JACQUARD *et al.* (2009), an increase in  $\text{CuSO}_4$  content in the medium has a positive effect on the density of plastids in the cells. The authors noted that for one of the examined genotypes, the presence of copper sulphate in the medium stimulated the accumulation of starch grains in microspores. However,  $\text{Cu}^{2+}$  also induces amyloplasts to differentiate into proplastids and chloroplasts. Such a mechanism may explain the significant increase of the GP regeneration we observed in this study. The same tendency was noted in other reports (WOJNAROWIEZ *et al.* 2002). However, the number of AP was also higher in the group with copper sulphate than in the control. Consequently, the GP:AP ratio remained identically independent of the type of induction media.

This study confirms that  $\text{CuSO}_4$  tends to improve the general plant regeneration. This was earlier noted in callus cultures and anther cultures of barley, and currently in barley isolated microspore cultures (NUUTILA *et al.* 2000; WOJNAROWIEZ *et al.* 2002; JACQUARD *et al.* 2009). In our study we did not confirm the previous reports suggesting that additional copper supplementation reduces albinism in androgenesis. This discrepancy may result from genotypes used, procedure or type of stress and should be studied further. Nonetheless, it seems essential to use  $\text{CuSO}_4$  enriched media in isolated microspore culture.

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