Plant Cell Tiss Organ Cult (2017) 130:443–447
DOI 10.1007/s11240-017-1232-5

RESEARCH NOTE

Effect of boron on microspore embryogenesis and direct embryo to plant conversion in *Brassica napus* (L.)

Pitchayapa Mahasuk[1] · Annika Stina Kullik[1] · Mohammed Cassim Iqbal[2] · Christian Möllers[1]

Received: 27 February 2017 / Accepted: 2 May 2017 / Published online: 5 May 2017

Abstract The NLN-medium has been successfully used, since 1982, for microspore culture in *Brassica napus* and other *Brassica* species. Changes to the media composition were restricted to carbohydrate and nitrogen sources and growth regulators while micro-nutrients have not been optimized. The NLN-medium contains boron at a concentration of 162 µM. Boron is required for diverse physiological and metabolic processes in the cell. This study investigated the effect of seven- and 13-fold increased boron concentration on the induction of embryos in microspore cultures of four genotypes of *B. napus*. A significant improvement of microspore embryogenesis was achieved by both enhanced boron concentrations in the NLN medium. No effect on the regeneration of embryo to plant conversion was observed.

Keywords Boron · Stress · Reactive oxygen species · ROS · Microspore embryogenesis · Direct embryo to plant conversion · *Brassica napus*

Ever since Robert Lichter (1982) developed the NLN-medium for microspore culture in oilseed rape (*Brassica napus)*, this medium has been widely accepted and has been used almost exclusively for the generation of haploid and doubled haploid plants. The NLN-medium is based on the medium of Nitsch and Nitsch (1967) which was developed for in vitro cultures of *Plumbago indica*. Lichter (1985) supplemented this basal medium with L-serine (100 mg/L) and L-glutamine (800 mg/L) and some phytohormones. The original medium used by Lichter (1985) contains 125 mg/L magnesium sulphate, even though the frequently used NLN-medium (e.g. offered by the company Duchefa; http://www.duchefa-biochemie.com) contains only 61 mg/L $MgSO_4$. From the published literature it is not clear, how much effort Lichter (1985) made to optimize the composition of the NLN-medium. The results of Gland et al. (1988) do not allow concluding which of the nine different tested culture medium variants are more suitable for the regeneration of a maximum number of embryos. Surprisingly, few studies were made to optimize the culture media composition for *Brassica* species. Hansen and Svinnset (1993) reported that refreshing the medium after 3 days enhances embryo yield which indicates that the concentration of minor medium constituents could be limiting embryo induction. However, the positive effect of replacing the culture medium after 24–48 h has also been explained by auto-toxic effects of freshly isolated microspores (Kott et al. 1988). Only recently Leroux et al. (2016) reported that the omission of cobalt and copper in the NLN-medium did not affect embryogenesis. However, omission of iron from the NLN-medium for 3 days led to a doubling of the numbers of regenerated embryos. One hypothesis for this positive effect of the short transient iron deficiency is that there is an iron-induced oxidative damage. Hence iron deficiency during the first days of culture may help to alleviate the stress caused by reactive oxygen species (Leroux et al. 2016; Mittler 2017).

Oilseed rape requires higher levels of boron than do other species (especially monocotyledonous species) and shows high sensitivity to B deficiency (Zhang et al. 2014;

Communicated by Alison M.R. Ferrie.

✉ Christian Möllers
cmoelle2@gwdg.de

[1] Department of Crop Sciences, University of Göttingen, Von-Siebold-Str. 8, 37075 Göttingen, Germany

[2] Plant and Environmental Sciences, National Institute of Fundamental Studies, Hantana Road, Kandy 20000, Sri Lanka

Eggert and Von Wirén 2016). Shoot regeneration and shoot length was significantly enhanced in kiwifruit (*Actinidia deliciosa*) shoot cultures on MS-medium with a 20-fold higher boron concentration of 2 mM compared to the standard MS-medium (Murashige and Skoog 1962; Sotiropoulos and Dimassi 2004). Although the NLN-medium contains with 10 mg/L (162 µM) already a relatively high H_3BO_3 concentration, a further enhancement of H_3BO_3 may positively affect microspore culture. However, the effect of increased H_3BO_3 concentration on microspore embryogenesis and direct embryo to plant conversion has not yet been investigated in *B. napus*. Direct embryo to plant conversion in oilseed rape was shown to be a bottleneck in plant regeneration, because embryos frequently tend to undergo secondary somatic embryogenesis (Möllers and Iqbal 2009). A cold treatment of the embryos was shown to significantly improve direct embryo to plant conversion (Cegielska-Taras et al. 2002). The main known function of B in plants is the establishment of borate esters with apiose residues of two rhamnogalacturonan II (RGII) molecules (Kobayashi et al. 1996). Sub-optimal supply of plants with boron may lead, amongst others, to altered cell wall and membrane formation, reduced anther development, fertility, pollen tube growth, seed germination and shoot development (Camacho-Cristóbal et al. 2008, 2015; Zhao et al. 2012; for a review see; Zhang et al. 2014). The cell wall Arabinogalactan protein (AGPs) profile has been suggested as an early marker for microspore embryogenesis in *B. napus* (El-Tantawy et al. 2013). Furthermore, Pandey et al. (2012) discuss a role for boron in the induction of somatic embryogenesis. The present research was undertaken, stimulated by the work of Sotiropoulos and Dimassi (2004), to study the effect of increased boron concentration in the NLN-medium on microspore embryogenesis and direct embryo to plant conversion in oilseed rape.

The experiment was performed with the four *B. napus* genotypes Express 617, ER1321, ER228 and the backcross (BC_1) genotype Express 617 × (ER228 × Express 617). Express 617 is an inbred line of the winter oilseed rape cultivar Express and ER1321 and ER228 are inter-varietal substitution lines derived from a cross between Express 617 and RS239 (Ecke et al. 2015). Growth of donor plants for microspore culture, bud sampling and microspore isolation was performed as described by Iqbal et al. (1994). Microspores were isolated from 48 buds for each experiment and genotype. After the microspores were finally washed, the microspore pellet was resuspended in 1.5 mL NLN-medium with 162 µM H_3BO_3 (Duchefa; http://www.duchefa-biochemie.com). This suspension was then equally divided (500 µl) to the three treatments: (i) Control NLN medium with 162 µM H_3BO_3, (ii) NLN medium with 1162, and (iii) 2162 µM H_3BO_3 (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany). The microspore suspension was pipetted into a 9 cm diameter Petri dish containing 11.5 mL NLN medium for each treatment, to give a total of 12 mL of medium per Petri dish. This was a single experiment. The Petri dishes were sealed with two layers of Parafilm and incubated in the dark for 2 days at 32 °C and then moved to 28 °C for 8 days. An additional 5 mL NLN-medium, with the same boron concentration, was added to each Petri dish 10 days after microspore isolation. Then, the Petri dishes were transferred onto a rotary shaker at 80 rpm under a photoperiod of 12 h for 14 days at 22 °C. Fifteen days after microspore isolation, 10 mL of the old medium was removed. If there were more than 200 embryos growing per Petri dish, a small part of the embryos (100–200 embryos) was collected on one side of the Petri dish and the embryos were carefully transferred with a scalpel to the new Petri dish containing 15 mL fresh medium with the same boron concentration. The original Petri dish with the remaining embryos was refilled with fresh medium. The total number of microspore-derived embryos (MDEs) was counted at day 28 after microspore isolation. Five independent experiments were performed.

The effect of the increased boron concentration in the NLN-medium on direct embryo to plant conversion was analyzed using the BC_1 genotype. To determine the direct embryo to plant conversion microspore derived embryos, 0.8–1 cm in length from the two boron treatments (1162 and 2162 µM) and the control, were transferred to sterile boxes with agar solidified Gamborg B_5 medium supplemented with 1 mg/L gibberellic acid (Gamborg et al. 1968; http://www.duchefa-biochemie.com). Each treatment, comprising of five boxes (replicates) with eight MDEs per box, was incubated in a light thermostat for 7 days at 2 °C in continuous darkness. Then, these boxes were transferred to a growth chamber room under a photoperiod of 12 h for 28 days at 22 °C. The percentage of direct embryo to plant conversion was calculated as the number of MDEs that developed shoots from the apical meristem without callusing or development of secondary embryos. The experiments were repeated thrice. Results obtained from the independent experiments per genotype and treatment was used for calculating the analysis of variance by using PLABSTAT software (Utz 2011). For the effect of boron on the number of MDEs and on the direct embryo to plant conversion, the experiments were considered as random. The percentage data for direct embryo to plant conversion were arcsin transformed.

The analysis of variance revealed highly significant differences for the effect of the genotype and the boron treatment (Table 1). The variance components show a predominant effect of the genotype, but the effect of the boron treatment was still high compared to the effect of the experiment and the interactions. The heritability was with 98% high and the genotype × treatment interaction

Table 1 Analysis of variance for the effect of the genotype (G), the boron treatment (T), the experiment (E) and their interactions on the total number of microspore derived embryos

Source	DF	MS	Var.cp	F	LSD_5
G	3	333,430	21,753	46.8**	62.2
T	2	212,419	10,265	29.8**	53.8
E	4	15,011	657	2.1^{+}	69.5
TG	6	13,311	1237	1.9	107.7
ETG	43	7128	7128		
Total	58				

$^{+,**}$Significantly different at p = 0.10 and p = 0.01, respectively (F-test, ANOVA)

Var.cp Variance components

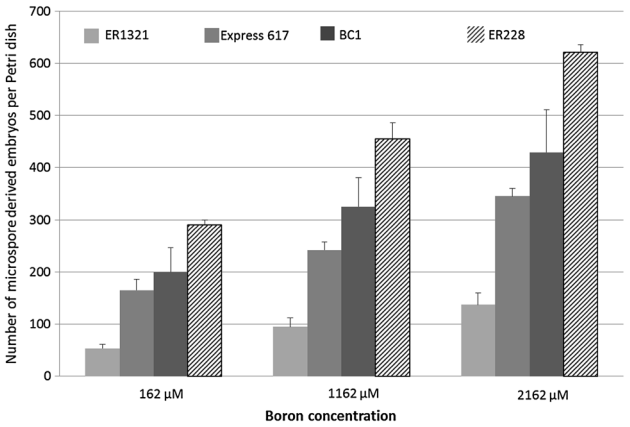

Fig. 1 Effect of the boron concentration on the number of microspore derived embryos of different genotypes (*data* represent means of five experiments). *Whiskers* indicate standard errors

was not significant, which indicates that the medium fortified with boron has a positive effect on all four genotypes (see also Figs. 1, 2). There was no morphological difference noted between the embryos grown at the three different boron concentrations. Express 617 has been reported before to have a comparatively low embryogenic potential (Klutschewski 2013; Ecke et al. 2015). Since an equal aliquot from the same final microspore suspension of each genotype was used for all treatments, the results are directly comparable. The cause of the positive effect of increased boron concentration on microspore embryogenesis is not clear. It could be that the boron concentration in the standard NLN-medium is sub-optimal. Furthermore, the higher boron concentration may have affected cell wall and membrane composition of the developing microspore (for a review see Zhang et al. 2014). Boron deficiency has recently been reported to be involved in inhibition of root cell elongation via an ethylene/auxin/ROS-dependent pathway in *Arabidopsis* seedlings (Camacho-Cristóbal et al. 2015). As mentioned above for iron, increased boron concentration may have alleviated stress caused by reactive oxygen species. This could have contributed to improved microspore embryogenesis. On the other hand, heat stress treatment is known to induce symmetrical first division in *B. napus* microspores (Lichter 1982; Iqbal et al. 1994). It cannot be excluded that the 7 (1162 µM) and 13-fold (2162 µM) higher boron concentration exerted stress on the microspores, which favored their symmetrical first division and further embryogenic development. Interestingly, Pandey et al. (2012) indicated that boron concentrations higher than 1 mM are considered as toxic for in vitro cultures for somatic embryogenesis. This obviously is not the case in the *Brassica* microspore embryogenesis system. Further studies have to show if lower concentrations of boron, as tested in this studies, have a similar positive effect of microspore embryogenesis. It remains open, whether combined treatments of heat shock, colchicine, boron and other compounds (e.g. Ahmadi et al. 2014, 2015) can lead to a synergistic effect on microspore embryogenesis. In somatic embryogenesis of *Daucus carota*, boron was not necessary for the induction of embryos but strongly influenced their normal development (Mashayeki and Neumann 2006). In barley, increased concentration of the micronutrient copper sulphate in anther culture has improved the frequency of responding anthers and of regenerated green plantlets in barley (Nuutila et al. 2000; Wojnarowiez et al. 2002; Jacquard et al. 2009). The positive effect of copper sulphate

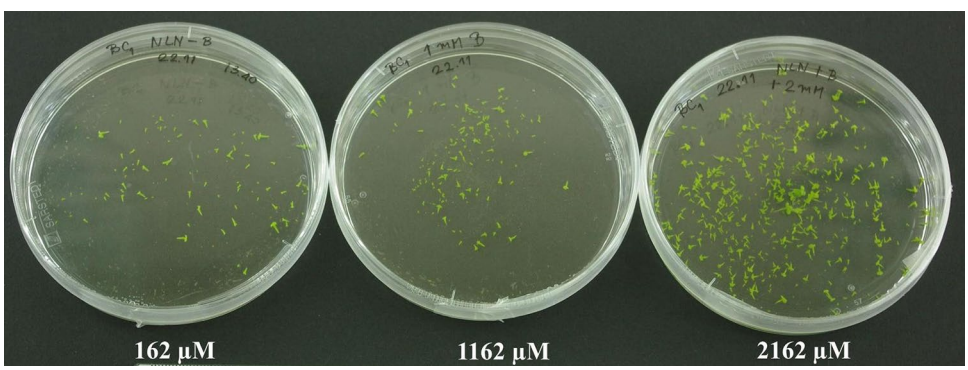

Fig. 2 Effect of different boron concentrations in the NLN medium on microspore embryogenesis of genotype BC1 at 17 days after microspore isolation

Fig. 3 Direct embryo to plant conversion of embryos in B5 media. Embryos were obtained from NLN medium with 162 µM (**a**), 1162 µM (**b**) and 2162 µM (**c**) boron concentration. **d** (insert) Some of the embryos failed to convert into plantlets and died

may rely on its ability to enhance microspore survival and synchronization of the first embryogenic division (Wojnarowiez et al. 2002), but this effect has not yet been investigated in *Brassica* microspore culture.

Since a latent shortage of boron was suspected to affect shoot development, the effect of increased boron concentration in the NLN-medium on the direct embryo to plant conversion was analyzed in the BC1 genotype. Following the cold treatment of the embryos a slight decline from 90.5% over 89.1–87.2% was observed for the three increasing boron treatments. However, these differences were not significant (data of the ANOVA not shown) and there was no visible difference between the plants derived from embryos regenerated on NLN-medium with different boron concentrations (Fig. 3a–c). A small number of the embryos failed to regenerate into plantlets and died, without showing any symptoms of bacterial or fungal contamination (Fig. 3d). In barley (*Hordeum vulgare*), the addition of the micronutrient zinc to the microspore culture induction medium increased the number of embryos up to 50% and similar to this study, had no effect on the regeneration of plants (Echavarri et al. 2008).

This is the first study on the effect of boron on microspore embryogenesis in *Brassica*. The findings from this study suggest that increasing the concentration of boron in the microspore culture induction medium of *B. napus* to 2000 µM increases embryogenesis in genotypes with low and high embryogenic ability. With the one genotype studied, there was no significant effect on the conversion of embryos to plants. It remains to be seen if the increase in boron concentration can have a synergistic effect with colchicine on embryogenesis in Brassica. A systematic study of other micro-nutrients of the NLN medium should be targeted to enhance embryogenesis in Brassica species, which could be unknown limiting factors and also their synergistic effects with other medium components.

Acknowledgements PM and MCMI thank the Erasmus-Mundus Action II (Experts-Sustain) program of the EU for research fellowships.

Author contributions CM and PM planned the experiment. PM, ASK and MCI performed the experiments. CM and PM analysed the data and all authors discussed the results. CM wrote the manuscript draft which was further improved by PM and MCI. All authors agreed to the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Ahmadi B, Shariatpanahi ME (2015) Proline and chitosan enhanced efficiency of microspore embryogenesis induction and plantlet regeneration in *Brassica napus* L. Plant Cell Tiss Organ Cult 123:57–65

Ahmadi B, Shariatpanahi ME, Ojaghkandi MA, Heydari AA (2014) Improved microspore embryogenesis induction and plantlet regeneration using putrescine, cefotaxime and vancomycin in *Brassica napus* L. Plant Cell Tiss Organ Cult 118:497–505

Camacho-Cristóbal JJ, Rexach J, González-Fontes A (2008) Boron in plants: deficiency and toxicity. J Integr Plant Biol 50:1247–1255

Camacho-Cristóbal JJ, Martín-Rejano EM, Herrera-Rodríguez MB, Navarro-Gochicoa MT, Rexach J, González-Fontes A (2015) Boron deficiency inhibits root cell elongation via an ethylene/auxin/ROS-dependent pathway in *Arabidopsis* seedlings. J Exp Bot 66:3831–3840

Cegielska-Taras T, Tykarska T, Szała L, Kuraś L, Krzymański J (2002) Direct plant development from microspore-derived embryos of winter oilseed rape *Brassica napus* L. ssp. oleifera (DC.) Metzger. Euphytica 124:341–347

Echavarri B, Soriano M, Cistué L, Vallés MP, Castillo AM (2008) Zinc sulphate improved microspore embryogenesis in barley. Plant Cell Tiss Organ Cult 93:295–301

Ecke W, Kampouridis A, Ziese-Kubon K, Hirsch A-C (2015) Identification and genetic characterization by high-throughput SNP analysis of intervarietal substitution lines of rapeseed (*Brassica napus* L.) with enhanced embryogenic potential. Theor Appl Genet 128:587–603

Eggert K, Von Wirén N (2016) The role of boron nutrition in seed vigour of oilseed rape (*Brassica napus* L.). Plant Soil 402:63–76

El-Tantawy AA, Solís MT, Da Costa ML, Coimbra S, Risueño MC, Testillano PS (2013) Arabinogalactan protein profiles and distribution patterns during microspore embryogenesis and pollen development in *Brassica napus*. Plant Reprod 26:231–243

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement of suspensions cultures of soybean root cells. Exp Cell Res 50:151–158

Gland A, Lichter R, Schweiger H-G (1988) Genetic and exogenous factors affecting embryogenesis in isolated microspore cultures of *Brassica napus* L. J Plant Physiol 132:613–617

Hansen M, Svinnset K (1993) Microspore culture of swede (*Brassica napus* ssp. rapifera) and the effects of fresh and conditioned media. Plant Cell Rep 12:496–500

Iqbal MCM, Möllers C, Röbbelen G (1994) Increased embryogenesis after colchicine treatment of microspore cultures of *Brassica napus* L. J Plant Physiol 143:222–226

Jacquard C, Nolin F, Hécart C, Grauda D, Rashal I, Dhondt-Cordelier S, Sangwan RS, Devaux P, Mazeyrat-Gourbeyre F, Clément C (2009) Microspore embryogenesis and programmed cell death in barley: effects of copper on albinism in recalcitrant cultivars. Plant Cell Rep 28:1329–1339

Klutschewski S (2013) Methodische Verbesserungen in der Mikrosporenkultur von *Brassica napus* L. PhD thesis Georg-August-Universität Göttingen. Ediss: http://hdl.handle.net/11858/00-1735-0000-0001-BC20-2

Kobayashi M, Matoh T, Azuma J (1996) Two chains of rhamnogalacturonan II are cross-linked by borate-diol ester bonds in higher plant cell walls. Plant Physiol 110:1017–1020

Kott LS, Polsoni L, Ellis B, Beversdorf WD (1988) Autotoxicity in isolated microspore cultures of *Brassica napus*. Can J Bot 66:1665–1670

Leroux BJG, Potin P, Larher FR, Bodin M (2016) Enhancement of embryo yield from isolated microspores of *Brassica napus* by early iron starvation. Plant Biotechnol Rep 10:483–486

Lichter R (1982) Induction of haploid plants from isolated pollen of *Brassica napus*. Zeitschrift für Pflanzenphysiol 105:427–434

Lichter R (1985) From microspores to rape plants: a tentative way to low glucosinolate strains. World Crops Prod Util Descr 11:269–277. ISSN 0166-6134. Ed. PAYS-BAS

Mashayekhi K, Neumann KH (2006) Effects of boron on somatic embryogenesis of *Daucus carota*. Plant Cell Tiss Organ Cult 84:279–283

Mittler R (2017) ROS are good. Trends Plant Sci 22:11–19

Möllers C, Iqbal MCM (2009) Doubled haploids in breeding winter oilseed rape. In: Touraev A et al. (eds) Advances in haploid production in higher plants. Vol 13. Springer, Berlin, pp 161–169

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Nitsch C, Nitsch JP (1967) The induction of flowering in vitro in stem segments of *Plumbago indica* L. I. The production of vegetative buds. Planta 72:355–370

Nuutila AM, Hamalainen J, Mannonen L (2000) Optimisation of media nitrogen and copper concentration for regeneration of green plants from polyembryogenic cultures of barley. Plant Sci 151:85–92

Pandey DK, Singh AK, Chaudhary B (2012) Boron-mediated plant somatic embryogenesis: a provocative model. J Bot. doi:10.1155/2012/375829

Sotiropoulos TE, Dimassi KN (2004) Response to increasing rates of boron and NaCl on shoot proliferation and chemical composition of in vitro kiwifruit shoot cultures. Plant Cell Tiss Organ Cult 79:285–289

Utz HF (2011) PLABSTAT—Ein Computerprogramm zur statistischen Analyse von pflanzenzüchterischen Experimenten. Version 3 A vom 14. Juni 2011. https://fsc.uni-hohenheim.de/fileadmin/einrichtungen/plant-breeding/plabstat_manual_ger.pdf (Site visited Feb 6, 2017)

Wojnarowiez G, Jacquard C, Devaux P, Sangwan RS, Clément C (2002) Influence of copper sulfate on anther culture in barley (Hordeum vulgare L.). Plant Sci 162:843–847

Zhang DD, Zhao H, Shi L, Xu FS (2014) Physiological and genetic responses to boron deficiency in *Brassica napus*: a review. Soil Sci Plant Nutr 60:304–313. doi:10.1080/00380768.2014.893537

Zhao ZK, Wu LK, Nian FZ, Ding GD, Shi TX, Zhang DD, Shi L, Xu FS, Meng JL (2012) Dissecting quantitative trait loci for boron efficiency across multiple environments in *Brassica napus*. PLoS ONE 7:14. doi:10.1371/journal.pone.0045215