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Isolated microspore culture of oat (*Avena sativa* L.) for the production of doubled haploids: effect of pre-culture and post-culture conditions

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Abstract

The production of doubled haploid (DH) plants from microspores is an important technique used in plant breeding programs and basic research. Although doubled haploidy efficiencies in wheat and barley are sufficient for breeding purposes, oat (*Avena sativa* L.) is considered recalcitrant. The objective of this project was to develop a protocol for the production of microspore-derived embryos of oat and further develop these embryos into fertile doubled haploid plants. A number of experiments were conducted evaluating the factors influencing microspore embryogenesis, i.e., donor plant conditions, pretreatments, media composition, and culture conditions. The initial studies yielded little response, and it was not until high microspore densities (10^6 microspores/ml and greater) were used that embryogenesis was achieved. Depending on the treatment, yields of over 5000 embryos /10^6 microspores were obtained for breeding line 2000QiON43. The doubled haploidy protocol includes: a 0.3 M mannitol pretreatment of the tillers for 7 days, culture in W14 basal medium with a pH of 6.5 – 7.5, a microspore density of 10^6 microspores/mL, and continuous incubation at 28°C incubation. The resulting embryos observed after 28 days were plated onto solidified W14 medium with 0.8 or 1.0 g/L activated charcoal. A colchicine treatment of 0.2% colchicine for 4 h resulted in conversion of 80% of the plants from haploid to doubled haploid. This protocol was successful for the production of oat microspore-derived embryos and doubled haploid green plants with minimal albinism. Doubled haploid seed was produced and planted for evaluation in a field nursery.

Keywords: *Avena sativa*, doubled haploid, microspore culture, oat

1 Introduction

2 Doubled haploid methodology to generate homozygous, true-breeding plants is used routinely in many plant
3 breeding programs. This technology has benefits for practical application (e.g. varietal development, mutagenesis,
4 and transformation) and basic research (e.g. genomics, biochemical, and physiological studies) (Dunwell 2010,
5 Ferrie and Möllers 2011). Doubled haploids are commonly produced using one of four methods: culture of anthers or
6 microspores (androgenesis), culture of unfertilized ovules (gynogenesis), interspecific or intergeneric crosses followed
7 by chromosome elimination, or pollination with irradiated pollen.

8

9 Oat (*Avena sativa* L.) is considered one of the more recalcitrant cereal crops with respect to doubled haploidy. Wide
10 hybridization with maize pollen (Rines 2003; Rines and Daheen 1990; Sidhu et al. 2006) generates doubled haploids
11 as has anther culture (De Cesaro et al. 2009; Kiviharju et al. 2000; Kiviharju et al. 2005; Ponitka and Slusarkiewicz-
12 Jarzina 2009), but methods are inefficient. Haploid embryo production using wide crosses has an efficiency of 0.8 –
13 6.7% (Sidhu et al. 2006) and for anther culture up to 30 green plants/100 anthers has been reported (Kiviharju et al.
14 2005). Haploid and doubled haploid plants have been regenerated from isolated microspore culture, but again at
15 very low frequency; two green plants and 15 albino plants were regenerated (Sidhu and Davis 2009).

16

17 Developing an efficient doubled haploidy protocol involves evaluating factors which influence induction of
18 embryogenesis and the regeneration of those embryos to plants (Ferrie and Caswell 2011). Prior to the culture of
19 microspores, the conditions in which the donor plants are grown, pretreatments given to floral buds, and the
20 developmental stage of the microspores selected for culture affect embryo production. The microspore isolation
21 method, the culture media, as well as the post-isolation conditions also influence the embryogenic response. This
22 paper describes several parameters affecting the production of microspore-derived embryos from isolated oat
23 microspores and the subsequent regeneration of green, doubled haploid plants.

24

Materials and methods

Germplasm

Genotype 2000QiON43 (LA9326E86) was selected for protocol development based on preliminary experiments and was provided by Stephen Harrison, LSU AgCenter, Louisiana, USA. 2000QiON43 is a non-released breeding line from the LSU AgCenter oat breeding program.

Donor plant conditions

Plants were grown in controlled environment chambers (20°C day/15°C night, 16 h photoperiod, 360 µmol m^{-2} s^{-1} light intensity). Seeds were planted in a soil-less mix (Sunshine #3/LG3-Sun Gro Horticulture) with approximately 1 g of slow release fertilizer (Nutricote 100 14-14-14; 14% nitrogen, 14% phosphorous, and 14% potassium) in a 15 cm pot. Plants were watered as required with a 0.88 g/L nutrient solution (20-20-20; N-P-K).

Original microspore culture protocol

Tillers were harvested when the panicle was 2.5 cm from the secondary leaf and placed in water for 7 - 10 days at 4°C. Panicles were then removed and spikelets containing microspores at the late uninucleate to early binucleate stage were placed into steel baskets for sterilization. The steel baskets were placed into a sterile beaker filled with 50% bleach (6.25% sodium hypochlorite) with a drop of Tween 20 surfactant and placed on a shaker for 10 minutes. The steel baskets containing the spikelets were then rinsed four times in sterile water for 5 minutes each. After sterilization, the panicles were put into a large blender cup (250 ml) containing 125 ml of 0.3 M mannitol (pH 5.8) and blended for 5 seconds on low speed and 7 seconds on high speed until homogenized. The homogenate was filtered through a sterile 90 µm Nitex screen into 50 mL centrifuge tubes and centrifuged at 489 g for 3 minutes. The supernatant was removed, the pellet was resuspended in 5 mL of 0.3 M mannitol, and the new suspension centrifuged as above. This was repeated 3 times. Prior to the last wash step, the microspore density was determined using a hemacytometer. The microspores were suspended in liquid culture medium (W14 basal medium unless otherwise specified; Ouyang et al. 1989) adjusting cell density as required in each experiment, plated in Petri dishes (100 mm x 15 mm), wrapped in Parafilm®, and incubated in the dark at the temperature outlined for each experiment. Embryos were counted after 4 weeks. Experiments were repeated three to 4 times, with a minimum of three replicate plates per treatment for each experiment.

Factors evaluated to enhance microspore embryogenesis in oat:

Microspore density

Five microspore densities (5×10^4, 1×10^5, 5×10^5, 1×10^6, 2×10^6 microspores/mL) were compared and evaluated for embryogenic response. Tillers were pre-treated in water for 7-10 days at 4°C and microspores were cultured in W14 basal medium and incubated continuously at 28°C. Microspores were plated in Petri dishes (35 x 10 mm) with 1 mL of culture medium.

Tiller pretreatment

Tillers were harvested as previously described and treated in water or 0.3 M mannitol for 7 days at 4°C. Microspores were isolated as described and cultured in W14 basal medium at densities of 1×10^6, 2×10^6 or 4×10^6 microspores/mL and incubated continuously at 28°C.

Culture temperature

Experiment 1: Tillers were pre-treated in 0.3 M mannitol for 7 days at 4°C. Microspores were isolated as described and incubated at 22°C, 24°C, or 28°C continuously in W14 basal medium at a density of 1×10^6 microspores/mL.

Experiment 2: Tillers were pre-treated and microspores isolated as in experiment 1. Microspores were incubated continuously at 22°C or 28°C, or 28°C for 1, 3, or 5 days then transferred to 22°C.

Donor plant conditions

Plants were grown in controlled environment chambers at temperatures of 20°C day/ 15°C night, or 10°C day/5°C night, harvested as described above, and used immediately or grown at 20°C day/ 15°C night, harvested and given a mannitol pretreatment (4°C for 7 days). Microspores were cultured in W14 basal medium at a density of 10^6 microspores/mL and incubated continuously at 28°C.

Media components

Five basal media were evaluated: W14 (Ouyang et al. 1989), NLN (Lichter 1982), modified FHG (Kasha et al. 2001), KFWC (Sidhu and Davies 2009), and TM (Tupý et al. 1991). Microspores were cultured at densities of 1 x 10^6 or 2 x 10^6 microspores/mL. A mannitol pretreatment was used for the tillers.

pH

Donor plants (grown at 20°C day/15°C night) and microspores were treated as described previously (mannitol pretreatment). Microspores were cultured in W14 basal medium with a pH range of 4.5 – 7.5, microspore density of 1 x 10^6/mL, and an incubation temperature of 28°C.

Embryo and plantlet regeneration

Embryos (approximately 1-2 mm) were transferred onto solid W14 regeneration medium (Kiviharju 2009), solid B5 (Gamborg et al. 1968) or solid MS (Murashige and Skoog, 1962) basal media all with 2% maltose and 0.3% phytagel and placed at 22°C continuously with a 16 h photoperiod (75 µmol m^{-2} s^{-1}). After 3 - 4 weeks, plantlets with green shoots were transferred to larger culture jars (Magenta™ GA7) with the same regeneration medium. To enhance root development and regeneration, activated charcoal (0.6, 0.8, or 1.0 g/L) was added to the W14 regeneration medium. Root development (i.e. root length, number of roots) was observed after 2 weeks. Once a well developed root system was established, plantlets were removed from the culture jars and the ploidy level of each microspore-derived plantlet was determined using flow cytometry.

Ploidy determination

A 1 cm^2 piece of young leaf was chopped with a razor blade in 400 µL ice cold nuclei extraction buffer (Cystain UV Precise, Partec) for 1 minute or until the leaf material was finely chopped. The suspension was filtered through a 30 µm screen to remove excess plant material leaving only plant cell nuclei for analysis. The extraction suspension was incubated for 5 minutes on ice, then 1.6 mL of nuclei staining buffer (Cystain UV Precise, Partec) was added to the suspension and the samples were incubated on ice for 1 minute. The Partec Cell Counter Analyzer (CCA) was used to determine the ploidy of the stained nuclei.

Chromosome doubling

The microspore-derived plantlets determined to be haploid were treated with colchicine for chromosome doubling. The roots and crown were soaked in a 0.1% or 0.2% colchicine solution for 1.5, 3, 4, or 5 h. After this time the plantlets were rinsed for 1 h in water, planted in 15 cm pots with commercial growing mix (Sunshine Mix #3/LG3), and covered with clear plastic cups to maintain a high relative humidity. The plastic cups were removed slowly once the plantlets were established. Doubled-haploid plants were grown in an controlled environment growth room (20°C day/15°C night, 16 h photoperiod, 360 µmol m^{-2} s^{-1}) and bagged individually to ensure self-fertilization. Resulting seed was planted in hill plots in a field nursery at the Crop Development Centre, University of Saskatchewan, Saskatoon, Canada in 2012.

Results and discussion

Many factors influence the embryogenic response of microspores, and these factors can be modified so as to generate microspore-derived embryos and subsequently doubled haploid plants. In our oat program, preliminary studies evaluated a number of genotypes, media compositions, pretreatments, and culture conditions (data not shown). From these experiments, genotype 2000QiON43 was selected for further experimentation. This was the same germplasm as Sidhu and Davis (2009) found most responsive.

Microspore density

From preliminary experiments evaluating a range of microspore densities (25,000 – 100,000 microspores/ml), cell division was observed in less than 1% of the microspores with no continued embryo development (data not shown). In the same preliminary experiments, higher densities (200,000 – 400,000 microspores/mL), more cell divisions and multi-cellular structures were observed, but no true-embryos (i.e. possessing both the scutellum and germ) developed. Very high densities (1 x 10^6, 2 x 10^6 microspores/mL) were then evaluated and found to be beneficial to embryogenesis (Table 1). With a density of 1 x 10^6 microspores/mL, a significantly higher number of embryos (or embryo-like structures, ELS) (1596/10^6 msp) was produced than any other density tested; this density also resulted in the highest number of large (1 – 2 mm in size with the scutellum and germ) embryos. There was little response at lower densities (5 x 10^4 and 1 x 10^5). Embryo development was not synchronous with each culture plate; although there were many ELS only a few were large enough (1 – 2 mm) for conversion to plantlets after 3 – 4 weeks (Table

1). Smaller ELS remaining in the plate did develop further, although not all became large enough for culture and plantlet development. Microspore density is an important factor in culture, as it affects both embryo quality and quantity. Optimal microspore density varies depending on the species. Whereas a low microspore density (1 x 10^4 microspores/ml) was beneficial for *Camelina sativa* (Ferrie and Bethune, 2011), a high microspore density (5 x 10^5) has been effective with barley (Kasha et al. 2003).

Tiller pre-treatments

Microspore-derived embryos and ELS were produced from both pre-treatments of tillers (water or mannitol). With both pre-treatments, a microspore density of 1 x 10^6 microspores (msp)/mL produced significantly more embryos and ELS than were produced at any other density. These results correspond with those from our previous microspore density studies. At each of the densities evaluated, there was no significant difference between the mannitol and water pre-treatment in terms of the number of ELS developed. However, mannitol pretreated tillers produced more 1-2 mm embryos than tillers treated with water (Table 2). A total of 48 green plantlets were produced from the mannitol pre-treatment whereas only 25 were produced from the water pre-treatment. There were very few albino plantlets produced. This 7 day pre-treatment is different from that of Sidhu and Davis (2009) who found a longer cold pre-treatment (6 – 9 weeks) beneficial in producing multicellular structures.

Culture conditions

In vitro embryogenesis can be influenced by the post isolation conditions of the microspores. Much research has focused on appropriate culture temperature and its duration, which varies among species. Culture temperature usually ranges from 24 – 27°C. For some species (e.g. *Brassica*), an elevated temperature (30 – 35°C) for 12 – 72 hours is required for embryo induction (Baillie et al. 1992; Ferrie 2003). A preliminary study in oat evaluating a range of temperatures determined that a heat shock of 32°C or 35°C was not beneficial as the oat microspores exhibited swelling but no further development. In contrast, microspores cultured at 28°C did produce multi-cellular structures. A further study evaluated three temperatures (22°C, 24°C, 28°C continuously). Embryos and ELS were produced from all temperature regimes, with significantly more ELS (1387 embryos/10^6 msp) being produced from the 28°C treatment (Table 3). No plantlets were regenerated.

Further experiments evaluated different heat shock periods (i.e. 1, 3, 5 days at 28°C followed by 22°C, continuous 28°C, or continuous 22°C). The longer the heat shock the more embryogenic the cultures. Microspore cultures incubated at 28°C for 5 days or continuously at 28°C were the most embryogenic (Table 4) with 2411 and 3061 embryos (ELS)/10^6 msp being produced, respectively.

Donor plant conditions

The environmental conditions in which donor plants are grown can influence embryogenic response. Healthy, vigorous donor plants are essential for successful microspore culture. Studies have shown that embryo/ELS yield can be affected by temperature, photoperiod, light intensity, and where the plant is grown (i.e. greenhouse, field, or growth chamber). For many species, growth of the donor plants at a low temperature (10°C day/5°C night) is beneficial (Baillie et al. 1992; Keller et al. 1987; Takahata et al. 1991). An experiment was designed to determine if the cold pretreatment of the tillers in mannitol (4°C for 7 days) could be replaced by growing the donor plants under cooler conditions (10/5°C). There was no significant difference in embryos or ELS/10^6 microspores between mannitol/cold pre-treatment and plants grown at 10/5°C; however, the mannitol pre-treatment produced more 1-2 mm embryos than the other two donor plant growing regimes (Table 5).

Basal media

The composition of the media plays a major role in embryogenesis. There are numerous studies evaluating the different components of media and their role in microspore culture. Initially, W14, a wheat and oat anther culture medium (Kiviharju 2009; Ouyang et al. 1989) was used. Utilizing two high microspore culture densities and a mannitol pre-treatment, five basal media were evaluated: W14, NLN [a medium used for microspore embryogenesis of *Brassica* species (Lichter 1982)], modified FHG [a barley anther culture medium (Kasha et al. 2001)], KFWC [used in oat microspore embryogenesis (Sidhu and Davies 2009)], and TM [a tobacco maturation medium (Tupý et al. 1991)]. Embryo-like structures were produced from all basal media and at both densities (Table 6). Although W14 medium at a density of 10^6 microspores/mL produced the most embryos/ELS (2067 embryos per 10^6 msp), at a density was 2 x 10^6 microspores/mL, KFWC medium produced more embryos/ELS than W14 at the same density.

pH

Sidhu and Davies (2009) reported induction of oat microspores when a high pH was applied (pH 8). In our experiments, medium pH and number of embryos/ELS were correlated (Figures 1, 2A; Table 7); however, at pH 6.5, 7, and 7.5 approximately 10% of the larger embryos developed callus (data not shown). The higher pH levels (7 and 7.5) are significantly better than the lower pH levels (4.5, 5, 5.8) for embryo/ELS production and also produced more large embryos than the lower pH levels.

Embryo culture regeneration

An important step in developing a doubled haploidy protocol is the conversion of embryos to plants (Figure 2 B, C). Observations from previous experiments indicated a low regeneration rate, which has also been reported for other cereals (Pauk et al. 2003, Pulli and Guo, 2003). Three basal media (W14, B5, MS) were compared and resulted in regeneration rates ranging from 10 – 21% (Table 8). The majority of the embryos/ELS did not develop or produced callus. The W14 medium produced 88% green plants along with 12% albinos. Use of the other basal media resulted in a higher frequency of albino plants.

Plantlet culture

With the addition of activated charcoal to the W14 medium, 0.6, 0.8, or 1.0 g/L, the embryos produced roots that were 9.7, 17.1, and 17.1 cm long, respectively, after two weeks. There was no root growth on W14 basal medium without activated charcoal. Overall, the total number of microspore derived green plantlets (Figure 2E) produced from genotype 2000QiON43 was 648 (91.7%) and the number of albino plantlets produced was 59 (8.3% albino). The low frequency of albino plants regenerated is beneficial as albinism can be a problem in many cereal crops (Torp and Andersen, 2009).

Ploidy determination

The ploidy, as described in the material and methods section, of 104 green plantlets was determined prior to chromosome doubling. Only 6% of plants had spontaneously doubled, while the majority were haploid (data not shown). Haploid plantlets (98) were treated with colchicine. Doubled haploid and non-haploid (haploid and mixaploid) were observed (Table 9). Plant survival rates for 0.1 % and 0.2 % colchicine were 97.9% and 93.6%,

respectively. The 4 hour treatment with 0.2% colchicine produced the highest conversion of haploid to doubled haploid plants (80%) and was concluded to be the best chromosome doubling treatment.

Field and greenhouse evaluation

Seed from 115 plants was evaluated in the greenhouse and in hill plots in the field (Figure 2F). Despite a wet growing season in 2012, the DH lines preformed well when compared to the control (parental) hill plots. Within the DH rows, plants were uniform as expected with DH lines. The plants tended to be shorter than the control, which has been observed in other DH lines grown in the field (Ferrie et al. 2011). This may also have been due to the background of the original material.

In conclusion, an improved doubled haploidy protocol that will yield sufficient doubled haploid plants for a breeding program was developed for oat as illustrated in Figure 2. Embryo/ELS yields of over 5000 embryos/10^6 microspores were obtained from genotype 2000QiON43. Plantlet regeneration from these embryos needs further improvement but those plantlets that did regenerate could be converted to doubled haploid plants with a colchicine treatment at a high rate of efficiency (80%). The protocol described here is different from previous published protocols as a short pre-treatment is used (7 days) and non-conditioned media is utilized both of which saves time and resources. Further research is required to expand the protocol to other oat germplasm.

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Figure 1: Effect of medium pH (4.5 – 7.5) on microspore-derived embryogenesis of oat genotype 2000QiON43.

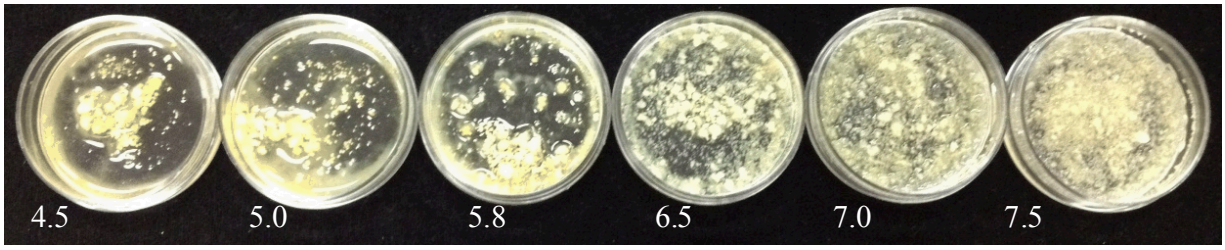

Figure 2: Doubled haploidy process in oat genotype 2000QiON43.
A. Microspore-derived embryos, B. Microspore-derived embryo on solid medium, C. Germinating embryo, D. Plantlet, bar = 1 cm, E. Haploid/doubled haploid plants in greenhouse, F. Doubled haploid (DH) plants in the field (Summer 2012). Bars for A, B, C = 1 mm.

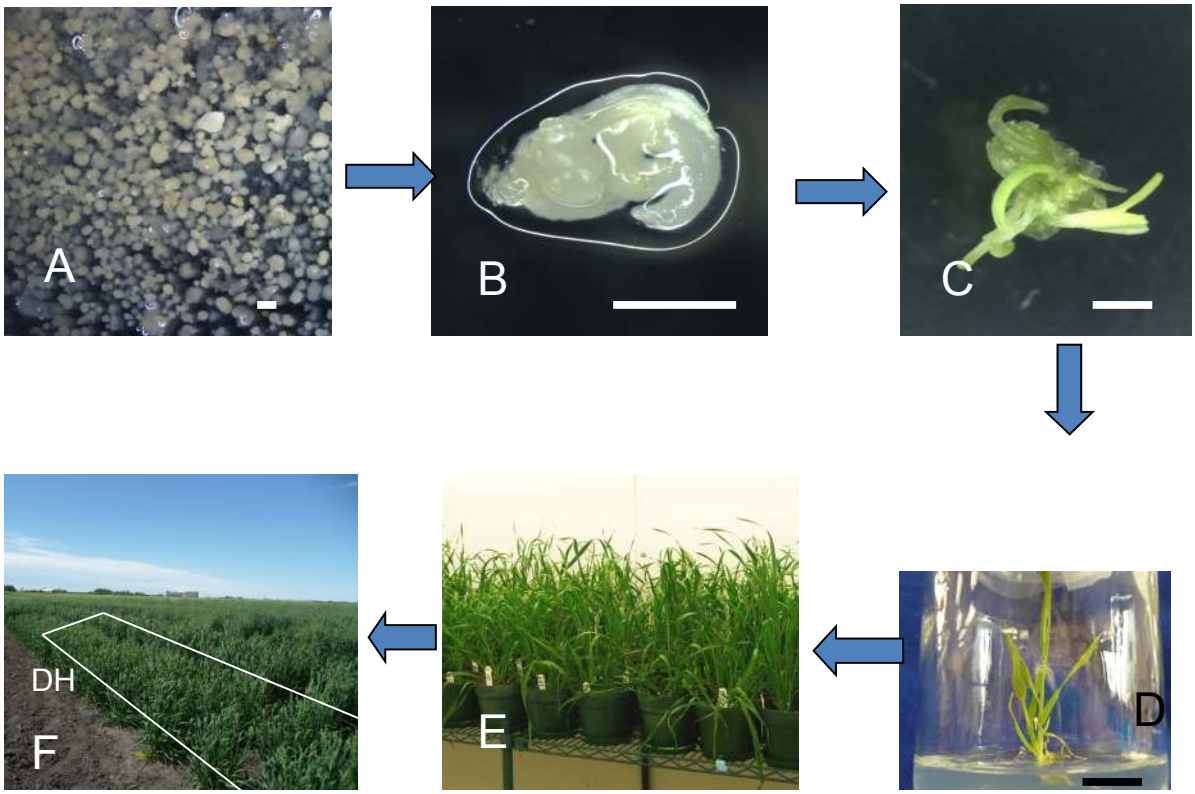

Table 1: Influence of microspore (msp) density on microspore embryogenesis of oat genotype 2000QiON43.

Microspore density msp/mL	Mean number of embryos/10^6 msp	Large embryos (1 – 2 mm) Total number (per 10^6 msp)
2 x 10^6	802 b	21 (2.6)
1 x 10^6	1596 a	110 (6.5)
5 x 10^5	608 bc	9 (0.8)
1 x 10^5	220 bc	7(0.2)
5 x 10^4	32 c	0 (0)

Means within a column followed by different letters in a column are significantly different at $P = 0.05$ level as determined by Duncan's multiple range test. Results are based on 3 experiments with 3 replicate plates per treatment per experiment.

Table 2: Influence of mannitol or water pretreatment on microspore embryogenesis of oat genotype 2000QiON43.

Microspore density (msp/mL)	Mean number of embryos/10^6 msp	Large embryos (1 – 2 mm) Total number (per 10^6 msp)	Number of green plantlets	Number of albino plantlets
Water pre-treatment				
1 x 10^6	2285 a	123 (2.9)	10	0
2 x 10^6	1558 b	114 (8.8)	9	1
4 x 10^6	1057 c	77 (12.8)	6	0
Mannitol pre-treatment				
1 x 10^6	2155 a	184 (4.5)	16	2
2 x 10^6	1544 b	40 (3.6)	0	0
4 x 10^6	1188 c	177 (29.5)	32	2

Means within a column and per pre-treatment followed by different letters in a column are significantly different at $P = 0.05$ level as determined by Duncan's multiple range test. Results are based on 3 experiments with 3 replicate plates per treatment per experiment.

Table 3: Comparison of incubation temperatures on microspore embryogenesis of oat genotype 2000QiON43.

Temperature (°C)	Mean number of embryos/ 10^6 msp	Large embryos (1 – 2 mm) Total number (per 10^6 msp)
22	297 b	0
24	659 b	0
28	1387 a	33 (2.8)

Means within a column followed by different letters in a column are significantly different at $P = 0.05$ level as determined by Duncan's multiple range test. Results are based on 3 experiments with 3 replicate plates per treatment per experiment.

Table 4: Effect of temperature (28°C) and duration of heat shock on microspore embryogenesis of oat genotype 2000QiON43.

Temp (°C) and duration (days)	Mean number of embryos/10^6 msp
22 – continuously	739 c
28 – 1 day	757 c
28 – 3 day	2135 b
28 – 5 day	2411 ab
28 - continuously	3061 a

Means within a column followed by different letters in a column are significantly different at $P = 0.05$ level as determined by Duncan's multiple range test. Results are based on 4 experiments with 3 replicate plates per treatment per experiment.

Table 5: Influence of donor plant conditions on microspore culture and embryo regeneration of oat genotype 2000QiON43.

Donor plant conditions/pre-treatment	Mean number of embryos/ 10^6 msp	Large embryos (1 – 2 mm) Total number (embryos /10^6 msp)
20/15°C Mannitol pre-treatment	2061 a	42 (3.0)
10/5°C No pre-treatment	1526 ab	5 (0.4)
20/15°C No pre-treatment	1157 b	26 (1.6)

Means within a column followed by different letters in a column are significantly different at $P = 0.05$ level as determined by Duncan's multiple range test. Results are based on 3 experiments with 3 replicate plates per treatment per experiment.

Table 6: Effect of basal medium on microspore embryogenesis of oat genotype 2000QiON43.

	Embryo production (mean number embryos /10^6 msp)	
Media composition	Original density 1 x 10^6 msp/ml	Original density 2 x 10^6 msp/ml
W14	2067 a	730 b
KFWC	1401 b	1232 a
FHG	767 c	540 b
TM	290 d	161 c
NLN	155 d	446 bc

Means within a column followed by different letters in a column are significantly different at $P = 0.05$ level as determined by Duncan's multiple range test. Results are based on 3 experiments with 3 replicate plates per treatment per experiment.

Table 7: Influence of basal medium pH on oat microspores genotype 2000QiON43.

pH	Mean number of embryos/1 x 10^6 msp	Large embryos Total number (per 10^6 msp)
4.5	2458 c	272 (22.7)
5	2811 bc	321 (26.8)
5.8	2977 bc	544 (45.3)
6.5	3959 ab	1551 (129.3)
7	5045 a	2029 (169.1)
7.5	5158 a	2081 (173.4)

Means within a column followed by different letters in a column are significantly different at $P = 0.05$ level as determined by Duncan's multiple range test. Results are based on 4 experiments with 3 replicate plates per treatment per experiment.

Table 8: Effect of basal medium on embryo conversion to plants in oat genotype 2000QiON43.

		Fate of embryos			
Basal Media	Total number of embryos cultured	Total number of embryos with no development or callus only	Total number of plantlets (% regeneration)	Total number of green shoots (% of total plants)	Total number of albino shoots (% of total plants)
W14	111	87	24 (21)	21(88)	3 (12)
B5	111	96	15 (14)	11(73)	4(27)
MS	112	101	11 (10)	9 (82)	2 (18)

Results are based on 4 experiments.

Table 9: Influence of colchicine concentration and duration on ploidy of oat plantlets derived from microspores.

Colchicine concentration and duration (h)	Haploid plantlets (%)	Double haploid plantlets (%)
0.1% Colchicine		
1.5 h	46.2	53.8
3 h	66.7	33.3
4 h	50.0	50.0
5 h	54.5	45.5
0.2% Colchicine		
1.5 h	53.8	46.2
3 h	41.7	58.3
4 h	20.0	80.0
5 h	45.5	54.5

Results are based on 1 experiment with a total of 101 microspore-derived plants.