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ORIGINAL ARTICLE

A fully in vitro protocol towards large scale production of recombinant inbred lines in wheat (*Triticum aestivum* L.)

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Abstract A bottleneck for genetic research and breeding of crop plants is the time taken to producing large pure line segregating populations so called recombinant inbred lines (RILs). One way to overcome this problem is through use of the single-seed-decent (SSD) process under in vitro conditions. A number of factors that may affect in vitro SSD approach of wheat including temperature, light duration and intensity, salt strength and carbohydrate concentration were investigated in this study. Under the in vitro conditions, 45 days per generation was achieved for an early flowering wheat genotype Emu Rock, allowing eight generations per annum; 58 days per generation was achieved for mid flowering genotypes, allowing six generations per annum. The results showed that a variation of growth environment before and after three-leaf stage allowed in vitro seed-set with a relatively short generation time. Specifically, the plantlets were first grown under 22 °C with a light intensity of 145 µmol m^{-2} s^{-1} (16 h d^{-1}) for 20 days (around three-leaf stage), and then moved to an environment of 28 °C and 500 µmol m^{-2} s^{-1} (20 h d^{-1}) light. The culture medium was 1/2 strength Murashige and Skoog (MS) with modification of adding ten times of extra KH_2PO_4 and 4% sucrose. The fully in vitro protocol resulted in 100% flowering rate and average seed set rate of 91.5% in Emu Rock and Zippy. It can be further fine-tuned to suit different genotypes and it has a potential for factory scale mass-production of RILs for genetic studies and practical breeding programs.

Keywords Wheat · *Triticum aestivum* L. · Fast generation cycling · Single-seed-decent · Flowering and seed-set in vitro

Introduction

Wheat is one of the most important crops worldwide. To satisfy the requirements for wheat breeding and genetic studies, a fast pure line production procedure is necessary. Recombinant inbred lines (RILs) are one of the most commonly used 'pure lines' (Ochatt et al. 2002). To obtain RIL populations, usually F6 is required for breeding purposes and F8 is required for genetic studies via single seed decent (SSD) to achieve the desired level of homozygosity (Liu et al. 2016). This process can take several years and limit the efficiency of breeding and genetic studies.

The use of anther or microspore culture for the production of doubled haploid (DH) lines has been proved successful for such purpose. However, the success rates for haploid induction in wheat is influenced by many factors (El-Hennawy et al. 2011), which limits its use in all genotypes for routine productions (Yermishina et al. 2004; Humphreys and Knox 2015). DHs are often produced after one generation of meiosis from F1 parental plants, resulting in limited recombination, which is an obvious disadvantage for breeding compared to SSD (Humphreys and Knox 2015).

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Using SSD approach, a two-step strategy, which forces flower differentiation through management of light, temperature, watering and potting mix in glasshouse, and cultures young embryos in vitro to circumvent the requirements for seed maturation, has been reported to dramatically shorten the lifecycles in crops (Croser et al. 2016; Liu et al. 2016; Mobini and Warkentin 2016; Ochatt et al. 2002; Ribalta et al. 2016; Yao et al. 2016; Zheng et al. 2013). However, the constant management of artificial growth environments such as light, temperature, water, nutrients, pest- and pathogen-control can be costly (Humphreys and Knox 2015). In addition, the grain sterilization and embryo dissection in every generation cycle for in vitro embryo culture can also become challenging when dealing with large-sized populations.

A one-step strategy which allows all generation cycles to be accomplished under in vitro conditions and the lifecycles of plants grown in optimized culture media and under automatic-controlled environment, is a promising alternative for large scale RIL production. The strategies have been successfully developed for pea, lentil, chickpea, common bean and faba bean (Ochatt et al. 2002; Ribalta et al. 2014; Mobini et al. 2015). Zhang and Tu (1986) also reported a preliminary protocol for wheat to flower and set seeds under in vitro conditions. However, this protocol had a long generation cycle of about 120–140 days, and a very low seed set rate for most of the plants tested, making it difficult to continue to proceed to the next generation cycle.

In order to achieve a large scale production of RILs, the important process is to accomplish the generation cycle under in vitro condition in a short period, and obtain the fertile embryos for the next generation. Shortening each generation cycle is critical to achieve fast generation in wheat using in vitro SSD method. Numerous factors, such as plant genotypes, culture medium composition (carbohydrate to nitrogen ratio, sugar, plant growth regulators, etc.), and environmental factors including temperature and photoperiod (Ochatt and Sangwan 2010; Ribalta et al. 2014; Smeekens et al. 2010; Ziv and Naor 2006), would affect the time of flower formation and seed-set in vitro (Vu et al. 2006).

To develop an applicable protocol for large scale production of RILs in wheat, a number of factors that may affect in vitro SSD approach of wheat, including temperature, light duration and intensity, salt strength and sugar content, were investigated in this study.

Materials and methods

Plant material

Three genotypes of wheat varieties; Emu Rock and Zippy representing typical early flowering and Kanata representing medium flowering were used in this study. Initially only one genotype Emu Rock was undertaken to develop the protocol and then the developed optimum protocol was further tested to the other two genotypes Zippy and Kanata.

Wheat embryos culture in vitro

Healthy seeds were surface sterilized by 70% (v/v) ethanol for 1 min, followed by 1% sodium hypochlorite solution for 10 min. The seeds were then rinsed three times with sterile deionised water and soaked in water overnight at room temperature (20–22 °C). Embryos were carefully dissected out from the seeds under aseptic conditions and placed on an embryo germination medium. Two media for one generation cycle were used (Table 1). The first medium is for embryo germination, which is a full strength MS medium (Murashige and Skoog 1962) containing 10% young coconut juice and 1% sucrose. The second medium is for plant development to flowering and setting seed. The embryos were firstly cultured on the germination medium. The reagents and preparation of medium plates were the same as described in Zheng et al. (2013). Plates containing the newly cultured embryos were kept on a bench top in a laboratory at room temperature of 22 °C with ambient lighting condition (no need of extra lighting or covers). Embryo

Table 1 Medium functions and MS medium modification with different concentrations of salts

Medium abbreviation	Medium function	Medium component
MS + 10% C + 1% S	Germination medium	Full strength MS salts adding 10% young coconut juice and 1% sucrose
MS + 2% S	Development medium	Control medium (full strength MS salts with 2% sucrose)
1/2 MS + 2% S	Development medium	1/2 MS salts with 2% sucrose
1/2 MS + 5 KP + 2% S	Development medium	Extra KH_2PO_4 850 mg L^{-1} (five times of KH_2PO_4 content in MS) in medium 1/2 MS + 2% S
1/2 MS + 10 KP + 2% S	Development medium	Extra KH_2PO_4 1700 mg L^{-1} (ten times of KH_2PO_4 content in MS) in medium 1/2 MS + 2% S
1/2 MS^m + 5 KP + 2% S	Development medium	Extra KNO_3 1800 mg L^{-1} and $MgSO_4$ $7H_2O$ 100 mg L^{-1} in medium 1/2 MS + 5 KP + 2% S
1/2 MS^m + 10 KP + 2% S	Development medium	Extra KNO_3 1800 mg L^{-1} and $MgSO_4 \cdot 7H_2O$ 100 mg L^{-1} in medium 1/2 MS + 10 KP + 2% S

germination usually took about 3 days. After 7 days, the germinated seedlings were transferred to the second development medium in the 150 × 70 mm polycarbonate containers containing 50 ml MS medium with different salt and sugar concentrations to select the optimum medium for flowering and setting seed. The containers were sealed with a screw cap (Sarstedt Australia Pty Ltd, Adelaide, Australia) with a 4 mm diameter hole covered by breathable membrane (Flora Laboratories, Melbourne, Australia).

Experimental design

In order to get the optimum conditions for wheat in vitro flowering and seed-set, the effects of following factors including environmental factors, salt and sugar contents of the plant development media were studied using genotype Emu Rock. Zippy and Kanata were tested after the suitable environmental factors and the best plant development medium were identified.

Environmental factors

The containers were placed under three different environments: (1) 22 °C constant temperature and 16 h d^{-1} photoperiod with light intensity of 145 µmol m^{-2} s^{-1} photon flux density (pfd); (2) 28 °C and 20 h d^{-1} photoperiod with 500 µmol m^{-2} s^{-1} pfd light intensity; (3) 22 °C and 16 h d^{-1} photoperiod with 145 µmol m^{-2} s^{-1} pfd light intensity until seedlings reached three-leaf stage, and then transferred into 28 °C with 500 µmol m^{-2} s^{-1} pfd light intensity (20 h d^{-1}). White fluorescent tubes were used as light sources.

Salt contents of the plant development media

Several development media with different concentrations of salts were investigated to screen the optimised medium to accelerate plant flowering and seed-set in vitro. The contents of salt were showed in Table 1.

Sugar contents of the plant development media

To evaluate the effect of sucrose and glucose contents on in vitro flowering and seed-set, the individual seedling were transferred to the previous optimised medium 1/2 MS^m + 10KP (Table 1) with 2% or 4% sucrose, or with 4% glucose while keeping other parameters unchanged.

Subculture of next generation embryos

Our previous research found that 8 days post-anthesis (dpa) was the earliest possible age to achieve embryo germination in wheat (Zheng et al. 2013). Once the in vitro cultured plants flowered and set seeds, 8 dpa embryos were removed from the young seeds and cultured on the same embryo germination medium as the first generation. One generation cycle time was calculated from embryos cultured on the medium to the next generation embryos cultured on the medium.

Statistical analysis

The experiments were repeated three times, with 10 seedlings per treatment for each replication. The results were expressed as means ± standard deviation (SD) of experiments. The significance of differences was carried out using Duncan's multiple range tests (DMRT) at $P < 0.05$.

Results

Effects of temperature and light on in vitro development of Emu Rock

Different combinations of temperature and light conditions were investigated for the Emu Rock seedlings cultured on MS medium with 2% sucrose (Table 2). The results showed that E1 treatment of 22 °C and 16 h d^{-1} photoperiod with 145 µmol m^{-2} s^{-1} pfd could advance the development of tillers. 60 days under in vitro culture, mean tiller number reached 5.2, and 30% plants could flower but only 10% plants set seed. However, E2 treatment with increased temperature and light intensity and duration (28 °C and 20 h d^{-1} photoperiod with 500 µmol m^{-2} s^{-1} pfd) obviously inhibited the developments of plants, and no plant could flower and set seeds.

Table 2 Effects of environmental factors on in vitro tiller development, flowering and seed-set in Emu Rock

Treatment[x]	Average number of tillers[y]	Flowering rate (%)	Seed set rate (%)
E1	5.2 ± 0.6a	30	10
E2	–	0	0
E3	2.8 ± 0.4b	50	30

E1: temperature 22 °C, light duration 16 h d^{-1}, and light intensity 145 µmol m^{-2} s^{-1}

E2: temperature 28 °C, light duration 20 h d^{-1}, and light intensity 500 µmol m^{-2} s^{-1}

E3: temperature 22 °C, light duration 16 h d^{-1}, and light intensity 145 µmol m^{-2} s^{-1} for 20 days, followed by 28 °C, 20 h d^{-1} and light intensity 500 µmol m^{-2} s^{-1}

[x]Plant culture medium used was MS + 2% sucrose

[y]Numbers followed by different letters (a or b) were statistically different at $P < 0.05$ by Duncan's multiple range test

The most favourable condition for the in vitro plants to flower and set seed was the E3 treatment, i.e. temperature 22 °C, light duration 16 h d^{-1} and light intensity 145 µmol m^{-2} s^{-1} pfd for 20 days (around the three-leaf stage) and then changed to 28 °C and 20 h d^{-1} light at 500 µmol m^{-2} s^{-1} pfd. The optimised temperature and light could increase flowering rate and seed-set rate to 50% and 30%, respectively, within 60 days.

Effects of salt and sugar modifications in media on in vitro flowering and seed-set of Emu Rock

In order to accelerate flowering and setting seed of the genotype Emu Rock, the effects of salt and sugar modifications in media were tested under the optimised environmental temperature and light situation, i.e. 22 °C with light duration 16 h d^{-1} and light intensity 145 µmol m^{-2} s^{-1} pfd for 20 days (around the three-leaf stage) and then changed to 28 °C with 20 h d^{-1} light at 500 µmol m^{-2} s^{-1} pfd (Table 3).

The number of days from sowing to flowering on the control medium MS was 52 days and one generation cycle was about 60 days, the seed-set rate was 30%. In order to shorten the generation cycle and increase the seed-set rate, the full strength MS medium was cut down to 1/2 MS and 5 or 10 times more KH_2 PO_4 were added to the 1/2 MS medium. The modifications in media stimulated flowering and shortened generation cycle 5 and 14 days, respectively, however, seed-set rates remained low (30%) and needed to be improved. Therefore, extra KNO_3 (1800 mg L^{-1}) and $MgSO_4$ ·$7H_2O$ (100 mg L^{-1}) were added to the 1/2 MS media with 5 or 10 times KH_2 PO_4 to adjust the balance between vegetative and reproductive development. The results showed that the salt modifications of adding extra 10 times KH_2 PO_4 and increasing the contents of KNO_3 and $MgSO_4$ in the 1/2 MS media resulted in a generation cycle of 46 days from sowing to flowering in Emu Rock and increased seed-set to 70%, which effectively shortened 7 days of the generation cycle and increased 40% of the seed-set rate compared to the control MS medium.

The effects of sucrose and glucose on wheat flowering and seed-set in vitro were also tested. The results showed that sucrose in the media could accelerate wheat plants flowering and seed-set in vitro (Table 3). Compared to 2% sucrose, 4% sucrose shortened the time about 7 days from sowing to flowering. On the contrary, 4% glucose delayed flowering time and decreased seed set compared with that of 4% sucrose. Finally, 1/2MS medium added with 10 times KH_2 PO_4, extra KNO_3 and $MgSO_4$ with 4% sucrose was the best medium that realized one generation cycle within 45 days with 90% plants set seeds (Fig. 1).

Responses of three wheat genotypes tested with the optimum protocol

Based on the optimum protocol established using genotype Emu Rock, two other genotypes Zippy and Kanata were also tested on the best medium and under the optimised environmental factors, varying temperature and light after the three-leaf stage. The results showed that the time from treatment to flowering for the early flowering genotypes of Emu Rock and Zippy was approximately 37 and 40 days, respectively, and the in vitro protocol realized 100% flowering and seed set above 90%. Each plant usually set one seed under the in vitro condition and the embryos dissected from the in vitro seeds were fertile and could germinate normally. Taking the 7 days embryo germination time into consideration, this protocol could produce one generation

Table 3 Effects of salt and sugar modifications in MS media on in vitro flowering and seed-set in Emu Rock

Medium[x]	Days to flowering[y]	Flowering rate (%)	Seed rate (%)	One cycle (days)[z]
MS + 2% S	52.3 ± 2.8a	50	30	60
1/2MS + 2% S	55.0 ± 0.9a	60	40	63
1/2MS + 5KP + 2% S	48.0 ± 1.9b	60	30	56
1/2MS + 10KP + 2% S	37.6 ± 1.1d	100	30	46
$1/2MS^m$ + 5KP + 2% S	49.5 ± 2.5ab	70	50	58
$1/2MS^m$ + 10KP + 2% S	45.3 ± 1.2c	90	70	53
$1/2MS^m$ + 10KP + 4% S	37.5 ± 1.7d	100	90	45
$1/2MS^m$ + 10KP + 4% G	45.4 ± 3.3c	80	60	53

[x]The composition of media were the same as shown in Table 1 except for the last two media which added 4% sucrose or 4% glucose(G), respectively

[y]Mean ± standard deviation; Numbers followed by different letters (a, b, c or d) were statistically different at $P < 0.05$ by Duncan's multiple range test

[z]Eight day old embryos could be removed from the green seeds and cultured on media to germinate. One generation cycle time was calculated from seeds/embryos cultured on the media to the next generation embryos cultured on the media

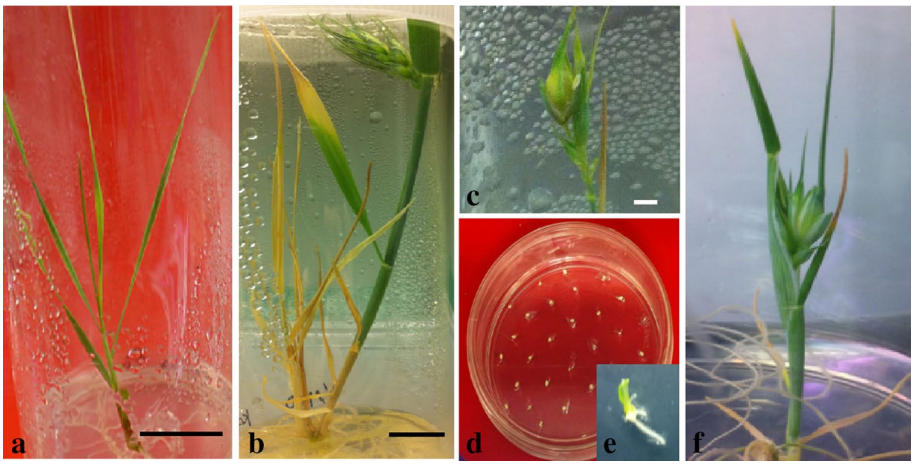

Fig. 1 Single-seed-decent (SSD) approach under in vitro conditions. **a** Plant cultured in jar (*bar* 1 cm); **b** earing stage of wheat plant (*bar* 1 cm); **c** setting seed (*bar* 1 mm); **d** embryos of immature seeds cultured in vitro; **e** germination of the next generation embryo; **f** earing stage of the next generation plant

Table 4 Responses of three wheat genotypes tested by the optimum protocol

Genotype	Days to flowering[x]	Flowering rate (%)	Seed set rate (%)	One generation cycle (days)	Cycles per annum
Emu Rock (early)	37.5 ± 1.7b	100	90	45	8.1
Zippy (early)	40.7 ± 3.5b	100	93	48	7.6
Kanata (mid)	50.5 ± 2.9a	86	73	58	6.3

[x]Mean ± standard deviation; Numbers followed by different letters (a or b) were statistically different at $P < 0.05$ by Duncan's multiple range test

cycle about 45 and 48 days for Emu Rock and Zippy, allowing 8.1 and 7.6 generations per annum. The mid-flowering genotype Kanata achieved 86% flowering and 73% seed set, spending 58 days for one generation cycle (Table 4).

Discussion

Using single-seed-decent (SSD) approach, a fully in vitro protocol for large-scale production of RILs in wheat were established and could achieved up to seven to eight generations per annum for the early flowering genotypes Emu Rock and Zippy on the 1/2 MS medium with added KH_2PO_4, KNO_3 and $MgSO_4$, and 4% sucrose under the optimised environmental conditions, temperature 22 °C, light duration 16 h d^{-1}, and light intensity 145 µmol m^{-2} s^{-1} for 20 days (around the three-leaf stage) and changed to 28 °C, 20 h d^{-1} and 500 µmol m^{-2} s^{-1}.

During the in vitro culture, it has been observed that the combined treatment of temperature 22 °C and lower light intensity 145 µmol m^{-2} s^{-1} pfd at early stage (before three-leaf stage) and higher temperature 28 °C and light intensity 500 µmol m^{-2} s^{-1} pfd at later stage (after three-leaf stage) could promote plants to flower and set seed. The result was consistent with previous reports that longer photoperiod promote early flowering for long-day plants. In legumes (long day plants), for example, it was reported that temperature and photoperiod were key factors in the transition to flowering (Nelson et al. 2010). It was also reported that the photoperiod of 20 h with a constant irradiance provided more rapid and uniform floral onset and seed set compare with the photoperiod of 13–14 h with fluctuant irradiance (Croser et al. 2016; Ribalta et al. 2016). Studies in rice have documented that photoperiod and high temperature acted synergistically on flowering time through the regulation of rice *FT* genes. High temperatures (28 °C) promoted flowering with increased *FT* expression (Song et al. 2012, 2013). Photoperiod and temperature affected *FT* protein output from the leaves, then *FT* protein moved to the shoot apex and induced flowering (Song et al. 2013; Blümel et al. 2015).

Under the in vitro growth conditions of this study, wheat plantlets generally developed tillers at three-leaf stage, which was around 20 days after culturing on the media. It was found that plants with fewer tillers were apt to flower and set seeds earlier compared to those with more tillers. The reason was probably because that the development of tillers would prolong the vegetative growth and delay flowering. Although the number of tillers in wheat is determined early in the life cycle which can be genotype dependent (Borras-Gelonch et al. 2012), it can also be influenced by temperature and light intensity (Bos and

Neuteboom 1998). This study found that increased temperature and light intensity and duration after three-leaf stage could apparently suppress tiller development. Sparkes et al. (2006) proposed that light interacting with leaf nitrogen content could determine the development of wheat tillers. This study was also found that salts modification of N:P:K in media could affect the number of tillers (data not shown). Liu et al. (2013) proved that inhibition of tillers could promote early maturation in rice. Therefore, supressing tillers development could contribute to the shortening of the generation cycle in wheat under in vitro condition.

Appropriate nutrition plays an important role on wheat development. The reduction of inorganic and organic salts in MS had a positive effect on flower induction (Vu et al. 2006). Zeng et al. (2013) suggested that high concentrations of nitrogen usually inhibited flowering and promoted vegetative growth whereas the use of 1/2 MS mineral medium or reduced nitrogen content could enhance in vitro flowering, especially low content of NH_4+ and high content of phosphorous could promote in vitro flowering (Kachonpadungkitti et al. 2001; Zeng et al. 2013). In the present study, a similar result was obtained that 1/2 MS with modification of some nutrition elements contributed to flowering than full strength MS. Moreover, the medium of 1/2MS with ten times of extra KH_2 PO_4 and increased KNO_3 and $MgSO_4$ could stimulate the plants to flower to a 100% rate and 10–15 days earlier, showing that suitable amount of nutrition would facilitate plant reproductive growth. This was in consistent with previous reports on *Chrysanthemum morifolium* (Liu et al. 2010), *Gnaphalium supinum* L. (Petraglia et al. 2014) and sunflower (Amanullah and Khan 2010).

Sugar is also an important factor to induce flower. Vu et al. (2006) reported that sucrose was the major factor needed in rose floral bud induction or initial development while other factor(s) were required to help the buds development fully at later stages of in vitro floral morphogenesis. In *Arabidopsis*, application of sucrose to the apical part of the plant stimulated flowering in complete darkness (Araki and Komeda 1993), giving further support to the hypothesis that sugars promote flowering (Susan 2005). Cha-um et al. (2012) believed that major carbohydrate resource from photosynthesis and sucrose-applied medium, might play a key role in carbohydrate signaling for flower initiation in rice because they observed that soluble sugars accumulated to higher levels in the leaves of flowering plants compared to non-flowering plants. In this study, both 2% and 4% sucrose in media could induce flowering and seed-set in vitro while 4% sucrose made plants flower 7 days earlier than 2% sucrose, supporting the report of high sucrose concentration promoting floral induction (Vu et al. 2006; Zeng et al. 2013). Meanwhile, 4% glucose delayed flowering and inhibited seed-set in vitro, suggesting sucrose was more suitable than glucose for in vitro fast generation of wheat.

Using the optimized environment, genotypes Emu Rock and Zippy achieved 45 and 48 days of one generation, 100% flowering rate and average seed set of 91.5% on the modified 1/2 MS medium with 10 times extra KH_2 PO_4 content and 4% sucrose. The embryos dissected from the in vitro seeds were fertile and could germinate and continue to develop to the next generation normally, without any albinism and deformity. Use the protocol developed in this study, close to 70% of time for one generation can be saved compared to the long generation cycle of 120–140 days reported by Zhang and Tu (1986). However, the performance difference in genotypes was observed, as the protocol was more suitable for early flowering genotypes and should be optimized for the mid and late genotypes to increase the percentage of flowering and seed set. Despite of this, the fully in vitro protocol developed in this study has a potential for fast mass-production of RILs in breeding programs and for genetic studies in wheat (*Triticum aestivum* L.).

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Author contributions YY, HL, ZL, GY conducted experimental design, data analysis and manuscript writing. PZ was involved in strategic experimental input. YY and HL conducted the in vitro experiments.

Compliance with ethical standards

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

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