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Factors affecting plant regeneration from immature inflorescence of two winter wheat cultivars

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Abstract

Inflorescence explants of two winter wheat cultivars, *Triticum durum* cv. Kızıltan-91 and *T. aestivum* cv. Bezostaja-01, were used to evaluate the effects of vernalization period of donor plants, callus age and medium composition on regeneration capacity. Donor plants were grown for 7 d and they were exposed to 4 ºC for 1, 2, 3, 4, and 5 weeks. The maximum inflorescence formation was observed as 79 % at 4 weeks and 73 % at 5 weeks of vernalization period for Kızıltan-91 and Bezostaja-01, respectively. Among 6 different callus induction and regeneration mediums, I_1-R_1 and I_3-R_3 have to be the best responding mediums for Kızıltan-91 and Bezostaja-01, respectively. In Kızıltan-91, calli induced from donor plants, vernalized for 3 weeks, showed a significantly lower regeneration capacity than counterparts vernalized for 4 and 5 weeks. The highest regeneration capacity of 69 % was obtained from 6-week-old calli produced from 4 weeks vernalized Kızıltan-91 donor plants. In contrast to Kızıltan-91 cultures, the effects of vernalization period and callus age on regeneration capacity were not significant in Bezostaja-01 cultures. The maximum numbers of tillers were obtained from 6- and 15-week-old calli for Bezostaja-01 and Kızıltan-91, respectively. In contrast to vernalization period of donor plants, callus age had no effect on seed number.

Additional key words: callus age, plant growth hormones, *Triticum aestivum*, *Triticum durum*, vernalization.

Introduction

Wheat, like other cereal species, shows a common recalcitrance and strong genotypic variation in tissue culture. A variety of explants sources have been used for obtaining wheat plants *in vitro* cultures. These are: isolated microspores (Mejza *et al*. 1993), shoot tips (Viertel and Hess 1996), mature embryos (Özgen *et al*. 1998, Delporte *et al*. 2001), immature embryos (Ozias-Akins and Vasil 1982,1983, Vasil *et al*. 1990, Fennell *et al*. 1996, Harvey *et al*. 1999, Pellegrineschi *et al*. 2004), leaf base (Wang and Wei 2004, Haliloglu 2006), and immature inflorescences (Ozias-Akins and Vasil 1982, Caswell *et al*. 2000, He and Lazzeri 2001). In cereals, immature embryos are considered as the best responding explants in culture due to their ability to produce embryogenic callus, readily followed by the production of large number of plants. Immature inflorescences have also been used for *in vitro* regeneration of other cereals, such as barley, rice, rye, maize (Barro *et al*. 1999). Advantages of using immature inflorescence tissue instead of immature embryo are that explants are harvested from younger plants reducing growth chamber requirements and physiological status of donor plants appears to have less influence on explants response in culture (Barro *et al*. 1999). Clear genotypic differences were stated in two studies (Maddock *et al*. 1983, Sharma *et al*. 1995), while Redway *et al*. (1990) observed no significant differences between cultivars.

Winter wheat cultivars usually have a greater vernalization requirement. Vernalization is determined by *Vrn* genes that are primarily located on group 5 chromosomes (Snape *et al*. 2001). Vernalization treatment might vary by genotype and ranged from 14 to - 52 d (Wang *et al*. 1995, Sharma and Mascia 1987, Özgen *et al*. 2001). Conventional vernalization, seedling vernalization, and direct explant vernalization are the most commonly used *in vitro* vernalization treatments (Sharma and Gill 1982). However, there were no data showing the effect of vernalization period on regeneration capacity, number of tillers and seeds in immature inflorescence derived wheat cultures.

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Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - dichlorophenoxyacetic acid; IAA - indole acetic acid; MS - Murashige-Skoog medium.

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The objective of the present study was to investigate the influence of vernalization period of donor plants and callus ages on regeneration capacity of calli, number of tillers and number of seeds produced from immature inflorescences cultures of two winter wheat cultivars.

Materials and methods

Two winter wheat cultivars [*Triticum durum* Desf. cv. Kızıltan-91 (durum wheat) and *Triticum aestivum* L. cv. Bezostaja-01 (bread wheat)], were grown in soil in greenhouse with supplementary light providing a 16-h photoperiod, with a day/night temperature of 20/18 ºC. On 7th day of growth plants were subjected to vernalization for 1, 2, 3, 4 and 5 weeks at 4 ºC at a 12-h photoperiod provided by 70 W fluorescent lamps giving approximately 150 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR). Plants were transferred back to greenhouse after vernalization and were fertilized once a week with 20:20:20 (N:P:K) liquid fertilizer supplemented with micronutrients. Tillers containing immature inflorescences were collected prior to the emergence of flag leaf which corresponds to 50 - 60 d for Kızıltan-91 and 80 - 90 d for Bezostaja-01. Tillers obtained from plants that were subjected to different vernalization periods were collected at the same time when inflorescence were between 0.5 - 1.0 cm in length. Surface sterilization was performed by sequential washing of the tillers in 70 % ethanol for 30 s and then 20 % sodium hypochlorite for 25 min, followed by three rinses in sterile distilled water. Immature inflorescences were isolated from tillers under stereo microscope and cut into approximately 1-mm long segments prior to culture.

Media with varying compositions were tested for callus induction and regeneration studies. The medium I_1: MS (Murashige and Skoog 1962) basal salts, 30 g dm^{-3} sucrose and 2 mg dm^{-3} dichlorophenoxyacetic acid (2,4-D) (Sharma *et al*. 1995), medium I_2: MS basal salts including Gamborg's B5 vitamins, 30 g dm^{-3} maltose and 2 mg dm^{-3} 2,4-D (Przetakiewicz *et al*. 2003), and medium I_3: MS basal salts including Gamborg's B5 vitamins, 30 g dm^{-3} maltose and 2 mg dm^{-3} *Picloram*. To evaluate their ability to initiate calli and to produce embryogenic tissues, 200 explants of 1 - 2 mm pieces were inoculated in callus induction media with 3 replications. For regeneration studies, three different media were used. They were medium R_1: ½ MS basal salts, 3 % sucrose, medium R_2: MS basal salts including Gamborg's B5 vitamins, 30 g dm^{-3} maltose, 1 mg dm^{-3} 6-benzylaminopurine (BA), 0.2 mg dm^{-3} IAA, and medium R_3: MS basal salts including Gamborg's B5 vitamins, 30 g dm^{-3} maltose, 1 mg dm^{-3} zeatin riboside, 0.1 mg dm^{-3} 2,4-D. The media were solidified with *Phytagel* (2.8 g dm^{-3}) and the pH was adjusted to 5.6 - 5.8 with NaOH prior to autoclaving. During preliminary studies which were conducted to test the effect of medium type on regeneration capacity, 6-week-old calli induced from 4 weeks vernalized explants were used. The best responding media, I_1-R_1 and I_3-R_3, were used for further studies for Kızıltan-91 and Bezostaja-01, respectively.

In order to determine optimum vernalization period, explants obtained from plants vernalized for 1, 2, 3, 4, and 5 weeks were collected at the same time. Forty tillers were used in each experiment for both cultivars and the experiments were replicated 5 times. Percentage of immature inflorescence formation for each vernalization period was determined by the number of immature inflorescence formed explants over total explants.

To evaluate the effects of vernalization period and callus age on regeneration capacity, calli produced from 3, 4, and 5 weeks vernalized explants were used. Since they could not produce sufficient number of immature inflorescence at a given time, 1 and 2 weeks vernalized explants were omitted. Explants (about 1 mm in length) were incubated at 25 ± 1 ºC in total darkness for 6 weeks for callus induction. Six-week-old calli were cut into two halves. One half was placed onto callus maintenance medium and kept at dark as done previously, while other half was placed into Petri dish containing regeneration medium and incubated in tissue culture room at 25 ± 1 ºC, 16-h photoperiod and PAR of 350 µmol m^{-2} s^{-1}. Regenerating plantlets were transferred into jars containing the same regeneration medium at 4-week intervals, whereas non-regenerating calli were kept in Petri dishes with fresh medium. For 15 weeks, explants were scored at every 4 weeks to determine the percentage of shoot producing calli and the mean number of shoots produced by each calli. The same procedure was repeated for 9-week-old calli and 12-week-old calli which were kept in callus maintenance medium. At the 15th week, all of the calli which were kept in callus maintenance medium were transferred onto regeneration medium. Two weeks after the transfer, jars containing plantlets were transferred into vernalization room. Since optimum vernalization period was determined to be 4 weeks in preliminary studies, plantlets were exposed to 4 °C at a 12-h photoperiod (150 µmol m^{-2} s^{-1} PAR) for 4 weeks. After the vernalization period, they were transplanted into soil (Fig. 1). The soil was previously autoclaved in order to prevent any contamination and weed formation.

Spike and seed analysis were conducted by using plants regenerated from Kızıltan-91. In order to evaluate the effects of vernalization period of donor plants and age of regenerating calli, seeds were scored by determining the number of seeds per regenerated plants. Randomly selected 50 regenerated plants were used for spike and seed analysis. Analysis of varience and Tukey's (HSD) pairwise comparison of means were conducted to compare different vernalization periods and callus ages on regeneration capacity, number of tillers and number of seeds.

Results

The highest inflorescence formation was observed in 4 (78 %) and 5 (73 %) weeks vernalized Kızıltan-91 and Bezostaja-01 plants, respectively (Fig. 2). Plants vernalized for 1 and 2 weeks had significantly lower inflorescence formation capacity than the others in Kızıltan-91 ($P < 0.001$). However, in Bezostaja-01, plants vernalized for 4 and 5 weeks had a significantly higher inflorescence formation capacity than 1, 2, and 3 weeks vernalized counterparts ($P < 0.001$). As a consequence of this study, explants of 1 and 2 weeks vernalized plants were omitted for further studies.

Genotype and medium composition were found to have a significant ($P < 0.05$) effect on regeneration capacity of calli (Table 1). When medium types were examined for their influence on regeneration, I_1-R_1 and I_3-R_3 were found to be the best responding couples for regeneration in Kızıltan-91 and Bezostaja-01 cultures, respectively. The maximum regeneration capacity was determined as 69.3 and 16.8 % for Kızıltan-91 and Bezostaja-01, respectively. Although, use of *Picloram* or 2,4-D did not influence the regeneration capacity of calli in Kızıltan-91 cultures, *Picloram* resulted with a higher

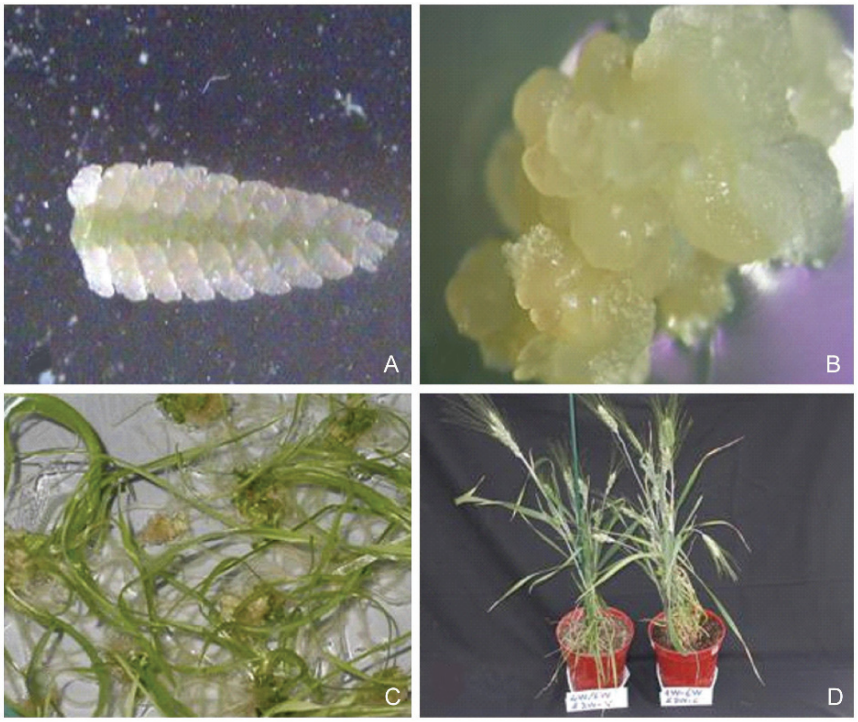

Fig. 1. Callus maintenance and plant regeneration from immature inflorescences of winter wheat (*T. durum* cv. Kızıltan-91). *A* - Immature inflorescence (about 1 cm length) used as explants; *B* - Four-week-old calli on I_1 medium; *C* - Plant regeneration on 2,4-D free MS medium from 6-week-old calli induced from 4 week vernalized explants; *D* - Regenerated plants with spikes after 10 weeks.

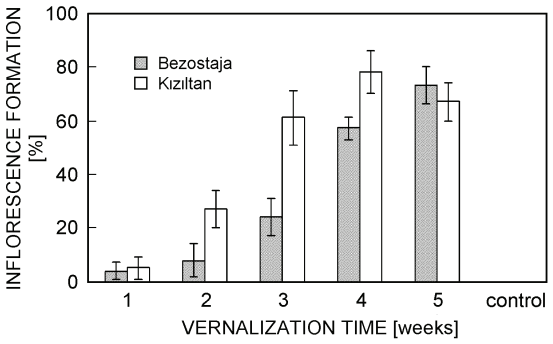

Fig. 2. Optimum vernalization period for cvs. Kızıltan-91 and Bezostaja-01. Means ± SE of five replicates with 40 explants per experiment.

regeneration potency of calli in Bezostaja-01 cultures (Table 1).

In Kızıltan-91 cultures, vernalization period and callus age had very important effects on regeneration capacity. When callus ages were examined in terms of regeneration capacity, it was seen that 6-week-old calli had higher regeneration capacity than the others. Likewise, vernalization time of explants have impact on regeneration capacity of calli. Calli induced from three weeks vernalized explants had lower regeneration capacity. There was no significant ($P > 0.05$) difference between regeneration capacities of calli induced from 4 and 5 weeks vernalized explants. The highest regeneration capacity was obtained as 69.34 % for

6-week-old calli induced from explants vernalized for 4 weeks. When vernalization period and callus age were examined for their influence on regeneration capacity of Bezostaja-01 cultures, no difference was observed in regeneration capacity among the different calli used (Table 2). The highest regeneration capacity was obtained as 26.8 % for 9-week-old calli induced from explants vernalized for 5 weeks. No difference was observed among calli that treated for different vernalization period in each cultivar in terms of number of tillers per regenerating callus. In contrast to vernalization period, callus age has influence on number of tillers in both cultivars. In Kızıltan-91 cultures, 15-week-old calli have significantly ($P < 0.001$) higher number of tillers than the others. However, 6 weeks old calli have higher number of tillers than the others in Bezostaja-01 cultures.

Seed analyses were conducted by using Kızıltan-91 cultivar in vernalized plants subjected to 4 ºC for 4 weeks and control plants not subjected to cold treatment after regeneration. According to average seed number, there was no effect of callus age on seed number in both control and vernalized plants. In vernalized plants, vernalization time of donor plants have an influence on average seed number. Plants produced by calli induced from 4 weeks vernalized donor plants had lower seed formation capacity than the others ($P < 0.05$). In contrast to vernalized plants, there was no effect of vernalization period on average seed number in control plants. Surprisingly, control plants that were not subjected to cold treatment after regeneration produced morphologically normal seeds like vernalized wheat plants. Average seed number was highest (9.3) in plants obtained from 9-week-old calli induced from 3 weeks vernalized donor plants.

Table 1. Effect of medium composition on regeneration capacity [%]. Six-week-old calli induced from 4 weeks vernalized explants were used. Columns with the same letter are not significantly different at the 0.05 level according to Tukey's pairwise test.

Medium	Kızıltan-91	Bezostaja-01
I_1-R_1	69.3^{a}	6.6^{c}
I_2-R_2	26.5^{b}	10.5^{c}
I_3-R_3	34.4^{b}	16.8^{d}

Table 2. Effects of vernalization period (3, 4, or 5 weeks) and callus age (6, 9, 12, and 15 weeks) on regeneration capacity of calli (R.C.) and number of tillers (N.T.). Means ± SE of five replicates with 80 - 100 explants per treatment.

Age [weeks]		Kızıltan-91 3	Kızıltan-91 4	Kızıltan-91 5	Bezostaja-01 3	Bezostaja-01 4	Bezostaja-01 5
6	R.C. [%]	28.9 ± 2.0	69.3 ± 2.5	59.3 ± 1.6	10.2 ± 0.7	22.8 ± 1.5	16.9 ± 1.2
	N.T. [callus^{-1}]	4.8 ± 1.1	4.2 ± 0.4	7.0 ± 1.4	5.8 ± 0.8	7.4 ± 0.4	6.0 ± 0.7
9	R.C. [%]	20.7 ± 1.5	57.3 ± 1.0	51.2 ± 1.2	7.1 ± 0.8	21.2 ± 1.2	26.8 ± 1.6
	N.T. [callus^{-1}]	2.5 ± 02	4.1 ± 1.0	4.2 ± 0.4	3.7 ± 0.6	5.4 ± 0.5	4.8 ± 0.8
12	R.C. [%]	12.2 ± 0.8	37.6 ± 1.6	34.3 ± 0.5	4.8 ± 0.4	10.7 ± 1.1	12.7 ± 0.5
	N.T. [callus^{-1}]	7.6 ± 1.9	6.3 ± 0.9	4.2 ± 1.7	3.3 ± 0.6	3.4 ± 0.5	3.0 ± 0.9
15	R.C. [%]	15.8 ± 0.9	43.3 ± 2.4	41.6 ± 0.7	4.3 ± 0.7	9.7 ± 0.9	11.8 ± 0.7
	N.T. [callus^{-1}]	6.6 ± 1.6	9.0 ± 0.7	4.5 ± 0.2	2.9 ± 0.6	2.2 ± 0.7	2.2 ± 0.4

Table 3. Effects of vernalization period (3, 4, or 5 weeks) and callus age (6, 9, 12, and 15 weeks) on average seed number per regenerating plant for control and vernalized Kızıltan-91 plants (4 weeks after regeneration). Columns and rows with the same letter are not significantly different at the 0.05 level according to Tukey's pairwise test.

Age [weeks]	Control plants 3	Control plants 4	Control plants 5	Vernalized plants 3	Vernalized plants 4	Vernalized plants 5
6	ND	3.3^{ab}	8.3^{b}	9.3^{bc}	1.5^{ab}	1.7^{ab}
9	5.0^{a}	4.4^{ab}	7.4^{b}	9.3^{bc}	3.8^{ab}	6.0^{abc}
12	0.1^{a}	5.6^{ab}	6.9^{b}	2.8^{ab}	4.0^{ab}	4.7^{ab}
15	2.7^{a}	4.7^{ab}	3.3^{b}	4.7^{ab}	3.0^{ab}	3.0^{ab}

Discussion

A reliable and efficient regeneration system is the fundamental point in biotechnological improvement of wheat and has been extensively investigated especially in terms of *in vitro* regeneration. In the present study, we propose a new regeneration system for winter wheat cultivars from immature inflorescence cultures. It has been shown that vernalization time of donor plant and callus age have a significant impact on regeneration capacity and number of tillers in Kızıltan-91 cultures. Immature inflorescence based regeneration system has several advantages over currently used regeneration system developed by Nehra *et al*. (1994). Donor plants can be planted more densely, and are grown for a shorter period of time and therefore, are less likely to suffer from

insect infestations (Caswell *et al*. 2000). The tissue sterilization and explant isolation procedures are also less time consuming than immature embryo based regeneration system.

Both callus induction and regeneration have been shown to be genotype dependent by several researchers (Fennell *et al*. 1996, Özgen *et al*. 1998) and these two parameters are also influenced by the components of the culture medium (He *et al*. 1989, Fellers *et al*. 1995). 2,4-D is the most widely utilized auxin in wheat cultures (Ozias-Akins and Vasil 1982, 1983, Redway *et al*. 1990, Sharma *et al*. 1995). None or other auxin-like compounds have been shown to induce somatic embryo differentiation and shoot formation. Germination of somatic embryos is usually induced on hormone-free medium or medium that contains low concentrations of auxin or of auxin and cytokinin (Carman 1995). In the present study we also tested the effect 2,4-D and *Picloram* in terms of callus induction and regeneration. Although *Picloram* has been reported to give rise to more regenerative callus than 2,4-D (Barro *et al*. 1999, He and Lazzeri 2001), this effect was only observed in Bezostaja-01 cultures.

Winter wheat cultivars usually have a greater vernalization requirement than many other winter cereals. However, this vernalization period may show variation from genotype to genotype. According to our knowledge, there was no data showing the optimum vernalization periods for Kızıltan-91 and Bezostaja-01. As a consequence of preliminary studies, optimum vernalization periods were found to be as 3 - 4 and 4 - 5 weeks for Kızıltan-91 and Bezostaja-01, respectively. We also checked the effect of vernalization period of donor plants on regeneration potency. Our results showed that explants, vernalized 3 weeks had a lower regeneration capacity than 4 and 5 weeks vernalized counterparts. To our knowledge, this is the first study investigating effect of vernalization period of donor plant on regeneration capacity of calli of inflorescence cultures of wheat. It has also been shown that age of callus significantly affect the regeneration capacity of calli. We observed that regeneration capacity of 6-week-old calli were significantly higher than 15-week-old ones. The reason for the effect of callus age might be due to some changes in the cellular polyamine contents (Khanna and Daggard 2001). Additionally, the effects of vernalization periods of donor plants and callus age on seed numbers were evaluated during this study. Our results showed that there was no effect of callus age on seed numbers for both control and cold treated plants. However, vernalization periods of donor plants had a influence on seed number for cold treated plants. Similarly, this is the first data showing the effect of callus age and vernalization periods of donor plants on seed number.

Currently, the established high frequency regeneration protocols are used to optimize particle bombardment and *Agrobacterium* based transformation protocols in local wheat cultivars.

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