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Efficient regeneration system from wheat leaf base segments

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Abstract

Efficient plant regeneration system from leaf base segments of wheat *(Triticum aestivum* L.) was developed. The factors affecting the callus formation and regeneration capacity of leaf segments of two genotypes; Bobwhite and Pavon 76, were investigated. The highest number of somatic embryos (SE) was obtained on Murashige and Skoog medium supplemented with 2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid + 1 mg dm^{-3} naphthalenacetic acid (14.7 SE per segment). Highest frequency of embryogenic callus (96 %) and somatic embryo formation (24.3 SE per segment) were achieved in the first segments. The highest plantlet regeneration was obtained after transfer of embryogenic calli to regeneration medium supplemented with 1 mg dm^{-3} kinetin (6.3 plantlets per segment).

Additional key words: auxins, cytokinins, somatic embryos, *Triticum aestivum* L.

Introduction

Wheat is one of the most important crops in the world and continues to be a difficult and important challenge for plant biotechnology. With the development of plant molecular biology and genetic engineering, its transformation has become one of the core issues in molecular breeding. First requirement for the successful application of biotechnology in crop improvement is to have efficient plant regeneration from cultured cells and tissues. Various explants such as anthers (Last and Brettell 1990, Haliloglu and Baenziger 2003a), isolated microspores (Mejza *et al*. 1993), immature embryos (Ozias-Akins and Vasil 1982, Vasil *et al*. 1990, An *et al*. 2000), inflorescences (Ozias-Akins and Vasil 1982, Maddock *et al*. 1983, Caswell *et al*. 2000), shoot tips (Viertel and Hess 1996) and mature embryos (Ozgen *et al*. 1996, Delporte *et al*. 2001) have been used for regeneration. Immature embryos have been determined to be most efficient tissue source to regenerate whole plants in large numbers (Ahloowalia 1982, Ozias-Akins and Vasil 1982) and are used extensively in genetic transformation studies of wheat (Weeks *et al*. 1993, Haliloglu and Baenziger 2003b).

The response of immature embryo to regeneration is commonly influenced by the conditions under which donor plants are grown (Vasil *et al*. 1993, Maës *et al*. 1996). Moreover, to grow donor plants for immature embryo culture is time consuming, labor intensive and expensive. Leaf segments are the most easily available donor material, since they can be grown *in vitro* and a short-term, frequent supply of explants can be provided (Jähne-Gärtner and Lörz 1996). The purpose of our study was to develop a simple, reproducible and reliable *in vitro* regeneration system from wheat leaf base segments. In the present study, the effects of genotype and development stage of leaves, various plant growth regulators and their combinations in callus initiation and regeneration medium on embryogenesis and regeneration from leaf bases were investigated. From this study, identified regeneration system will be used for *Agrobacterium* mediated genetic transformation experiments.

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Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MES - 2-[morpholino]ethanesulfonic acid); MS - Murashige and Skoog medium; NAA - naphthalenacetic acid; REG - regeneration medium

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Materials and methods

Mature seeds of two wheat (*Triticum aestivum* L.) cultivars, Bob White and Pavon 76, were used as starting material. The seeds were surface-sterilized with 70 % ethanol for 5 min and with sodium hypochlorite for 15 min and then thoroughly rinsed with sterile water. Seeds were germinated under light on wetted filter paper in Petri dishes. Four leaf segments from leaf base to tip (referred as 1 to 4) and each 3 - 4 mm in length, were dissected from leaf base of 3- to 4-d old seedlings. Segments 1 - 4 were compared for their embryogenic callus induction efficiency. Explants were cultured on the callus induction medium at 25 °C in the dark, and 30 d later, the callus induction, and somatic embryo formation for each segment were measured. Callus development during induction and initiation was periodically monitored. A modified MS (Murashige and Skoog 1962) medium containing MS macro and micro salts, thiamine HCl 0.5 mg dm^{-3}, pyridoxine HCl 2.5 mg dm^{-3}, nicotinic acid 2.5 mg dm^{-3}, myo-inositol 500 mg dm^{-3}, glycine 10 mg dm^{-3}, MES 1.95 g dm^{-3} and sucrose 30 g dm^3 was used for routine callus induction and growth. Media with varying concentrations of different plant growth regulators were tested for callus induction. They were medium A: 2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) + 1 mg dm^{-3} naphthaleneacetic acid (NAA); medium B: 2 mg dm^{-3} 2,4-D + 1 mg dm^3 kinetin; medium C: 2 mg dm^{-3} picloram (4-amino-3,5,6-trichlor-opicollinic acid) + 1 mg dm^{-3} kinetin, medium D: 2 mg dm^{-3} indole-3-acetic acid (IAA) + 1 mg dm^{-3} kinetin; medium E: 1 mg dm^{-3} NAA + 1.5 mg dm^{-3} kinetin, and medium F: 2 mg dm^{-3} indole-3-butyric acid (IBA) + 1 mg dm^{-3} kinetin. The media were solidified with *Phytagel* (2 g dm^{-3}) and the pH was adjusted to 5.6 - 5.8 with NaOH prior to autoclaving.

For regeneration, calluses were transferred onto regeneration medium. Regeneration medium was full strength MS salt and vitamins with three different cytokinins, which were kinetin 1 mg dm^{-3} (REG1), 6-benzylaminopurine (6-BAP) 1 mg dm^{-3} (REG2) or zeatin 1 mg dm^3 (REG3) with 30 g dm^{-3} sucrose solidified with 0.25 % *Gelrite* at pH 5.8. They were kept under 16-h photoperiod (fluorescent light tubes providing irradiance of 62 μmol m^{-2} s^{-1}) and temperature of 25 °C. Thirty days after transfer to regeneration media the number of shootlets *ca*. 1.5 cm in length or longer was recorded. When regenerated plantlets had well-developed roots, they were transferred to soil.

Experiment was carried out using completely randomized design (CRD) with 4 replications of 25 segments per plate. Callus induction and somatic embryo formation for each segment were measured after 30 d in induction medium. The callus formation percentage was calculated as the number of segments forming callus as a percentage of cultured segments. The percentage of embryogenic calli was evaluated as the number of segments producing somatic embryos as a percentage of the total number of segments induced. The number of plantlets per cultured segment resulted from the total number of regenerated plantlets divided by the number of cultured segments. Analysis of variance was used to compare the means. *SAS/PC* statistical program was used for all computations (*SAS Institute Inc*., Cary, USA).

Results

Callus formation was started after 7 - 10 d from the cut ends of the leaf base segments (Fig. 1*A*,*B*) and followed by development of embryogenic calli (Fig. 1*C*). Callus induction and somatic embryo formation were influenced by the type and concentrations of growth regulators, the position of the leaf segments. Callus formation and somatic embryo formation was observed in all callus initiation media. However, some plant growth regulators promoted the callus induction and somatic embryo formation better than the others. Highest somatic embryogenesis was observed in medium A (14.7 SE per segment). Medium A, containing higher auxin concentration compared to other media, triggered higher embryogenic callus formation as well as somatic embryo production. Therefore, medium A is used in further optimization experiments. Low embryo formation was observed when media C, D and E were used for callus induction (4.2, 3.6 and 2.8 SE per segment, respectively, Table 1).

First segments in two genotypes based on all investigated callus initiation media were found to be very

Table 1. Effects of 6 callus initiation media on somatic embryogenesis. Means ± SE, $n = 400$.

Medium	Growth regulators [mg dm^{-3}]	Number of somatic embryos [$explant^{-1}$]
A	2,4-D + NAA (2+1)	14.7 ± 2.4
B	2,4-D + kinetin (2+1)	11.3 ± 1.8
C	picloram + kinetin (2+1)	4.2 ± 2.3
D	IAA + kinetin (2+1)	3.6 ± 2.5
E	NAA + kinetin (1+1.5)	2.8 ± 1.2
F	IBA + kinetin (2+1)	7.4 ± 0.8

responsive explants for *in vitro* culture. Analysis of variance showed the significant differences among tested leaf base segment based on embryogenic callus formation ($P < 0.01$) and plantlets regeneration ($P < 0.01$). Highest frequency of embryogenic callus (96 %) and somatic embryo formation (24.3 SE per segment) were achieved in the first segments. Embryogenic callus formation in the second segment was decreased to half of what first segment performed (52 %). Our results showed that the embryogenic potential of the wheat leaf tissue is determined by the position of the leaf segments. When explants were getting far from the basal tissue, callus formation and consequently plantlets regeneration were decreased. Only enlargement of explant was observed in the third and the forth segments. In addition, no somatic embryo and plantlets regeneration was formed.

Two genotypes were employed in our study. Callus formation and somatic embryo formation were observed in both genotypes. Based on results obtained from this study, plant regeneration was succeeded in both genotypes. Genotype Bobwhite produced average 7.21 SE per segment used. In addition, this went up to 17.2 embryos per responsive segment and percentage of plantlets regeneration was 82.5 %. On the other hand, genotype Pavon 76 produced 6.98 SE per segment used, which was lower than Bobwhite genotype. When somatic embryo formation per responsive segment was considered, frequency was higher in Pavon 76 than Bobwhite

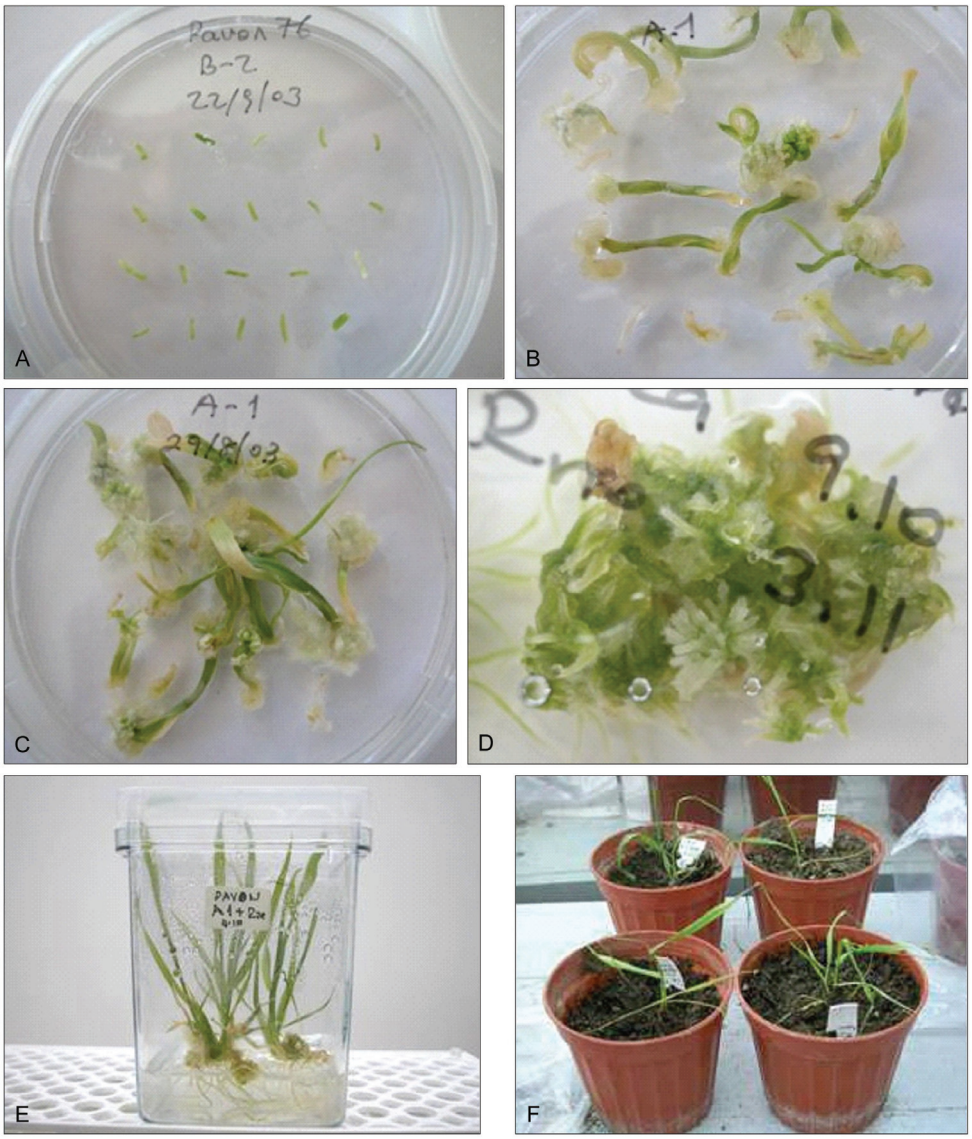

Fig. 1. Somatic embryogenesis and regeneration of plantlets from the first basal segment of wheat leaves: *A* - leaf base segments on callus initiation medium, *B* - callus formation on callus initiation medium, *C* - formation of embryogenic callus from the first basal segment, *D* - shoot regeneration from embryogenic calli, *E* - elongation of regenerated plants on regeneration medium, *F* - regenerated plants in the soil.

(18.4 embryos per responsive segment). Pavon 76 showed 79.4 % plantlet regeneration. These differences between genotypes were not statistically significant.

To increase regeneration efficiency, three cytokines were investigated on regeneration medium. They were kinetin (1 mg dm^{-3}), BAP (1 mg dm^{-3}) and zeatin (1 mg dm^{-3}). Plant regeneration was observed in all regeneration media. Numerous green patches appeared over the surface of embryogenic calli within 5 d followed by the regeneration of shoots and roots simultaneously after 10 d of transfer of calli to regeneration medium (Fig. 1*D*). The highest plant regeneration was obtained in regeneration medium (REG1) containing MS + 1 mg dm^{-3} kinetin (6.3 plantlets per segment) and followed by regeneration medium (REG3) containing MS + 1 mg dm^{-3} zeatin with regeneration capacity of 3.6 plantlets per segment. MS + 1 mg dm^{-3} BAP medium (REG2) performed the lowest plant regeneration (1.8 plantlets per segment). Based on the results obtained from this study, plant regeneration generally follows the somatic embryogenesis path.

When the regenerated plantlets had well-developed root system (Fig. 1*E*), they were transferred to soil in growth chamber (Fig 1*F*). Plants regenerated from leaf base segments formed spike and spikelets as normal plants. No albino plant was observed *in vitro* originated plants. They were all fertile and morphogenetically normal.

Discussion

Leaf base segments in cereals serve as an excellent system to investigate competence of dedifferentiated cells due to the presence of a basal meristem (Wernicke and Brettell 1980, 1982). The regeneration potential of these explants can also be exploited for raising transgenics as has been achieved in oats (Matsuda *et al*. 1998). In the present study, efforts have been directed towards obtaining high regeneration efficiency from wheat basal segment cultures.

Only first and second leaf base segments produced satisfactory amounts of somatic embryos, and calli derived from the first basal segment were most potent for plant regeneration. Similar phenomenon has been observed for other cereals such as oat (Chen *et al*. 1995, Gless *et al*. 1998), barley (Becher *et al*. 1992) and wheat (Wernicke and Milkovits 1984, Rajyalakshmi *et al*. 1991). Whereas, third and forth segments did not form any callus and produce any plant, which was in accordance with an earlier notion that a decreasing gradient of response of callus formation from the base to the apex exists in leaves of cereals (Chen *et al*. 1995, Rajyalakshmi *et al*. 1991). Some researchers attempted to explain the existence of such gradient by variation in the location of the cells in the cell cycle, from the leaf base to its apex (Dolezelova *et al.* 1992).

It is well known that plant growth regulators plays crucial role in *in vitro* regeneration (D'Onofrio and Morini 2003/4, 2005). Our results also showed that the presence of 2,4-D in initiation medium was important for callus induction and somatic embryo formation from leaf base segments of wheat. Use of cytokinins in combination with auxins to induce somatic embryogenesis in callus cultures has been reported for cereals (Bhaskaran and Smith 1990, Gaspar *et al*. 1996). Whereas, our results showed that composition of cytokinin in callus induction medium could not increase the regeneration frequency. Similar results were also reported in oat (Gless *et al*. 1998) and wheat (Wernicke and Milkovits 1984).

Regeneration from wheat explants is considered to be highly genotype dependent (Wernicke and Milkovits 1984, Bajaj 1990, Maheshwari *et al*. 1990, Vasil and Vasil 1999). This aspect hampers wheat genetic improvement programs that are strongly dependent upon tissue culture procedures. However, the two genotypes of *T. aestivu*m, Bobwhite and Pavon 76, exhibited similar callusing and regeneration response, this can be explained by either the regeneration protocol developed is relatively free of genotype and cultivar constraints, or both genotypes are good for *in vitro* regeneration. Second explanation is more acceptable since Bobwhite is a model genotype for immature embryo culture and Pavon 76 is commonly used in anther culture studies for haploid plant production. Regeneration efficiency of the basal segment calli is comparable to that reported from mature and immature embryos (Bajaj 1990, Vasil and Vasil 1999). The protocol developed is also season-independent as seedlings, the explant source, are available throughout the year.

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