Plant Cell, Tissue and Organ Culture **77:** 245–250, 2004.

Effect of 2,4-dichlorophenoxyacetic acid and NaCl on the establishment of callus and plant regeneration in durum and bread wheat

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Received 30 September 2002; accepted in revised form 23 September 2003

Key words: 2,4-D, embryogenesis, NaCl, plant regeneration, wheat

Abstract

Optimal callus induction and plant regeneration were obtained in bread and durum wheat by manipulating the NaCl concentration in the induction medium. Immature embryos from a high regeneration line of spring wheat (*Triticum aestivum* L.), 'MPB-Bobwhite 26', and an elite durum wheat (*Triticum turgidum* var. *durum* L.), 'Mexicali', were cultured in E3 induction medium consisting of Murashige and Skoog (MS) medium, 2.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 2% sucrose and 0.9% Bacto agar. The treated embryos were transferred to E3 liquid medium supplemented with various levels of 2,4-D and NaCl. Incubation on medium containing 2.5 mg l^{-1} 2,4-D for 45 days produced callus and plant regeneration in 'MPB-Bobwhite 26', but lower callus yield and plant regeneration in 'Mexicali', indicating that 2,4-D alone was not sufficient for callus induction and plant regeneration in this durum variety. Callus yield and regeneration frequencies were higher in 'Mexicali' embryos that were incubated in media containing 2 mg l^{-1} 2,4-D and 2 mg l^{-1} NaCl. The presence of NaCl in the medium beyond the initiation phase was detrimental to plant regeneration. The use of NaCl in the callus formation could form the basis for improved transformation of durum wheat varieties.

Introduction

In bread wheat, many tissues have been used for the induction of morphogenic cultures. These tissues vary in their ability to regenerate whole plants; however, immature embryos are regarded as among the most suitable explants (Benkirane et al., 2000; Menke-Milczarek and Zimny, 2001). Durum wheat, compared to bread wheat, has not been extensively investigated with respect to plant regeneration from *in vitro* culture.

The conditions under which donor plants are grown can affect the morphogenic response of immature embryos (Arzani and Mirodjagh, 1999; Caswell et al., 2000; Delporte et al., 2001; Harvey et al., 2001) and transformation (Barro et al., 1998; Folling and Olesen, 2001; Weir et al., 2001; Wright et al., 2001; Pastori et al., 2002; Pellegrineschi, 2002b). Water stress can enhance somatic embryogenesis in durum wheat (Pellegrineschi et al., 2002a). The highest induction frequency of somatic embryogenesis was achieved from immature embryo cultures derived from water-stressed plants. Withholding water for 2–3 days from the mother can enhance embryogenic induction. However, this method requires relatively elaborate greenhouse facilities, which are not always available. Based on the observation that water stress can enhance somatic embryogenesis, we increased the osmolarity of the induction medium with various concentrations of NaCl and evaluated the effects on induction of embryogenic cultures.

Table 1. Effects on somatic embryo induction in durum wheat cultivar 'Mexicali' in the presence of 2,4-D and NaCl[a]

Concentration ($mg\,l^{-1}$)		15 days treatment		33 days treatment	
2,4-D	NaCl	Average number of embryogenic calli	Average number of plants developed	Average number of embryogenic calli	Average number of plants developed
Control	–	19.5 ± 1.5 kl[b]	13 ± 0.5 f	21.5 ± 6 ikl	14.5 ± 2 g
1	0	16 ± 3.5 l	10 ± 0.5 h	16 ± 14 ijklm	5 ± 0 j
1	1	60 ± 1.5 g	20 ± 0.5 d	21.5 ± 4 ikl	25 ± 1 f
1	3	135 ± 6 c	60 ± 2 a	29 ± 9 i	15 ± 0.5 g
1	5	125 ± 3.5 d	20 ± 0.5 d	24 ± 7.5 ik	15 ± 0.5 g
1	7	55 ± 1.5 h	15 ± 0.5 e	30.5 ± 9.5 i	25 ± 1 f
2	0	21 ± 1.5 k	13 ± 0.5 f	23.5 ± 13.5 ik	12.5 ± 1.5 h
2	1	185 ± 6.5 a	15 ± 0.5 e	120 ± 10 h	55 ± 1.5 c
2	3	125 ± 3.5 d	45 ± 1.5 b	160 ± 10 g	65 ± 2 b
2	5	120 ± 3 e	15 ± 0.5 e	360 ± 11.5 d	75 ± 1 a
2	7	155 ± 5.5 b	25 ± 1 c	600 ± 19 a	25 ± 1 f
3	0	15 ± 9.5 kl	11.5 ± 1.5 g	200 ± 7 f	12 ± 1 h
3	1	115 ± 3 e	10 ± 0.5 h	395 ± 12.5 c	30 ± 1 e
3	3	45 ± 3 i	5 ± 0 i	330 ± 10.5 e	75 ± 3.5 a
3	5	65 ± 1.5 f	0 ± 0 k	410 ± 13 c	45 ± 1.5 d
3	7	60 ± 2 g	20 ± 0.5 d	560 ± 17.5 b	45 ± 1.5 d
4	0	10 ± 0.5 m	0 ± 0 k	10.5 ± 3.5 klm	6.5 ± 0.5 j
4	1	25 ± 1 j	5 ± 0 i	12 ± 4 klm	15 ± 0.5 g
4	3	45 ± 1.5 i	0 ± 0 k	15.5 ± 5 ijklm	0 ± 0 k
4	5	65 ± 1.5 f	0 ± 0 k	23.5 ± 7.5 ik	10 ± 0.5 i
4	7	25 ± 0.5 j	0 ± 0 k	7.5 ± 2.5 l	0 ± 0 k
5	0	5 ± 1 n	0 ± 0 k	7.5 ± 3 lm	5 ± 0 j
5	1	55 ± 1.5 h	0 ± 0 k	9 ± 3 klm	10 ± 0.5 i
5	3	5 ± 0 n	1 ± 0.5 jk	14 ± 4.5 jklm	0 ± 0 k
5	5	4 ± 1.5 n	2 ± 0.5 j	9 ± 3 klm	0 ± 0 k
5	7	5 ± 1.5 n	2 ± 0.5 j	1.5 ± 0.5 m	0 ± 0 k

[a] Calli (15 or 33 days after induction) were cultured for 20 days in liquid medium containing the indicated concentration of 2,4-D and NaCl. Average number of embryogenic calli (15 days) least significant difference (LSD) = 3.6656; average number of embryogenic calli (33 days) LSD = 15.67; average number of plants developed (15 days) LSD = 1.1587; average number of plants developed (33 days) LSD = 1.9367.
[b] Means with the same letter are not significantly different.

Materials and methods

Preparation of plant material

The durum wheat cultivar 'Mexicali', and bread wheat cultivar 'MBP-Bobwhite 26', were grown in a screenhouse with solar radiation levels between 1000 and 1200 $Jm^{-2}\,h^{-1}$, and temperatures ranging between 18 and 25 °C. Spikes were harvested 12–16 days after pollination to obtain immature seeds. Seeds of each of the cultivars were sterilized with a 6% solution of commercial bleach (Cloralex), by stirring in a 200 ml sterile beaker for 20 min. The sterilized seeds were rinsed two times with sterile deionized water. Immature embryos, 0.8 and 1.0 mm long, were aseptically excised with a scalpel. The zygotic meristem was excised and the isolated scutella placed with the scutellum facing up on semi solid E3 medium in 100 mm × 20 mm plastic Petri dishes.

Culture media and experimental design

Somatic embryogenesis induction

Approximately 2500 explants for each wheat variety were used for embryogenic culture induction. All embryos initially were cultured on E3 medium consisting of MS (Murashige and Skoog, 1962) plant growth medium, 40 $mg\,l^{-1}$ thiamine HCl, 0.15 $g\,l^{-1}$ L-asparagine, 20 $g\,l^{-1}$ sucrose, 9 $g\,l^{-1}$ Bacto agar (Difco), and 2.5 $mg\,l^{-1}$ 2,4-D (Sigma) (Table 1). Prior to autoclaving, the pH was adjusted to 5.7.

For each treatment, 50 immature durum wheat and 50 bread wheat embryos were placed on E3 callus induction medium for 15 or 33 days. Calluses that developed from the treated embryos were transferred to Petri dishes (3 cm diameter, Sigma) containing 1 ml liquid E3 medium with various 2,4-D concentrations (1.0, 2.0, 3.0, 4.0 and 5.0 mg l^{-1}) in factorial combination with various NaCl concentrations (0.0, 1.0, 3.0, 5.0 and 7.0 mg l^{-1}). E3 callus induction medium containing 2.5 mg l^{-1} 2,4-D without NaCl was used as a control. The embryos were incubated for 20 days at 25–26 °C at 50 rpm. Subsequently, the embryos were transferred to regeneration medium with the same concentration of 2,4-D and NaCl as the liquid treatment and evaluated for somatic embryo and plant development (Figure 1).

Plant regeneration

The regeneration method was adapted from Pellegrineschi et al. (2002a, b) and used to compare the regeneration efficiency of the treated immature embryos. The measurement of the treatment effects on somatic embryogenesis and plant regeneration were measured by calculating the average number of embryogenic cultures that developed and the average number of whole plants recovered, regardless of how many per callus, and the total number of embryos treated. Five repetitions, with 100 immature embryos each (50 durum and 50 bread wheat), were used for each treatment. The average number of embryogenic cultures was calculated by dividing the total number of independently developed calluses by the total number of embryos treated. The average number of plants produced was calculated by dividing the total number of plants developed by the total number of embryos treated.

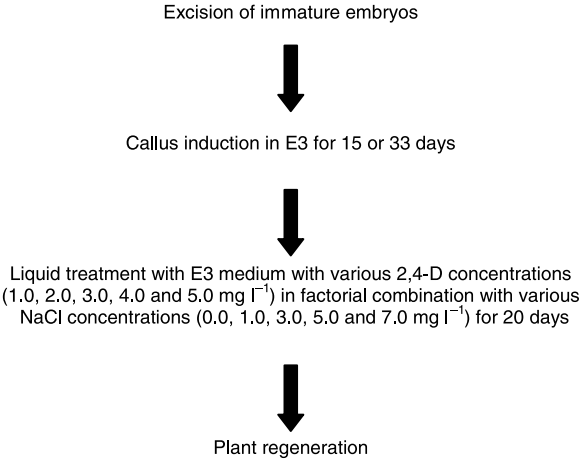

Figure 1. Flow chart of the experimented design.

Embryogenic cultures were transferred to 60 mm × 15 mm Petri dishes containing 3.0 ml of MS liquid regeneration medium without hormones. They were maintained in the growth room at 26 °C under a photoperiod of 16/8 h light/dark conditions at a light intensity of 200 Jm^{-2} h^{-1}.

Statistical analysis

An analysis of variance of the three factors (concentration of 2,4-D and NaCl, and time) and their two-way and three-way interactions was performed using SAS (Littell et al., 1996).

Results and discussion

Embryogenic culture induction and somatic embryo development

After preculture of zygotic embryos on E3 with 2.5 mg l^{-1} 2,4-D, embryogenic cultures were transferred to media with 0.0, 1.0, 3.0, 5.0 and 7.0 mg l^{-1} NaCl in combination with 1.0, 2.0, 3.0, 4.0 and 5.0 mg l^{-1} 2,4-D for 1 week. The callus initiation rate was 100% in all treatments. After 20 days, callus reached 2–3 mm^3 in volume and both embryogenic and non-embryogenic calluses were observed.

Embryogenic callus was greenish-white to white, smooth, with spherical globular embryos. Non-embryogenic callus was white, watery and friable with large, highly vacuolated cells. The different treatments were scored on the 20th day after culture on induction medium. The response of cultures varied depending on the composition of the medium (Tables 1 and 2). In treatments, the embryos developed more than one independent embryogenic culture. Each independently induced embryogenic culture was counted as one (Tables 1 and 2).

Somatic embryo germination and plantlet regeneration

For embryogenic culture induction in 'Mexicali', E3 medium supplemented with 2 mg l^{-1} 2,4-D and 1 mg l^{-1} NaCl and 2 mg l^{-1} 2,4-D and 7 mg l^{-1} NaCl gave the best results. Increased embryogenic culture

Table 2. Effects on somatic embryo induction in bread wheat cultivar 'MPB-Bobwhite 26' under the same conditions described for the cultivar Mexicali[a]

Concentration ($mg\,l^{-1}$)		15 days treatment		33 days treatment	
2,4-D	NaCl	Average number of embryogenic calli	Average number of plants developed	Average number of embryogenic calli	Average number of plants developed
Control	–	60 ± 1 nm[b]	48 ± 0.5 a	72.5 ± 3.5 n	49 ± 1 a
1	0	41.5 ± 7.5 op	41.5 ± 0.5 c	33.5 ± 1 o	32.5 ± 0.5 d
1	1	125 ± 4 gh	20 ± 0.5 gh	535 ± 16.5 a	6.5 ± 0 l
1	3	130 ± 4 g	15 ± 0.5 j	340 ± 10.5 de	9.5 ± 0.5 h
1	5	120 ± 4 h	20.5 ± 0.5 g	435 ± 13.5 b	8 ± 0.5 ij
1	7	250 ± 8 a	16.5 ± 0.5 i	220 ± 7 i	10 ± 0.5 gh
2	0	63.5 ± 2.5 lm	46.5 ± 0.5 b	68.5 ± 2 n	39 ± 0.5 b
2	1	70 ± 2 kl	21.5 ± 0.5 f	330 ± 10.5 e	7.5 ± 0 jk
2	3	200 ± 6.5 bc	21.5 ± 0.5 f	255 ± 8 g	10 ± 0.5 gh
2	5	205 ± 6.5 b	22 ± 0.5 f	355 ± 11 c	10 ± 0.5 gh
2	7	180 ± 5.5 d	19.5 ± 0.5 h	145 ± 4.5 l	7 ± 0 kl
3	0	47 ± 4.5 o	37 ± 0.5 d	43 ± 4 o	37.5 ± 0 c
3	1	55 ± 1.5 n	36.5 ± 0.5 d	315 ± 10 f	11 ± 0.5 ef
3	3	75 ± 2.5 k	13.5 ± 0.5 k	240 ± 7.5 h	10.5 ± 0.5 fg
3	5	180 ± 5.5 d	8.5 ± 0.5 m	350 ± 11 cd	4.5 ± 0 m
3	7	85 ± 2.5 j	23.5 ± 0.5 e	175 ± 5.5 k	11.5 ± 0.5 e
4	0	7.5 ± 4 q	15 ± 0.5 j	14 ± 1.5 p	5 ± 0 m
4	1	205 ± 6.5 b	8.5 ± 0.5 m	175 ± 5.5 k	8.5 ± 0.5 i
4	3	195 ± 6 c	5 ± 0 n	360 ± 11.5 c	5 ± 0 m
4	5	150 ± 4.5 e	15 ± 0.5 j	190 ± 6 j	0 ± 0 o
4	7	75 ± 2.5 k	20 ± 0.5 gh	110 ± 3.5 m	2.5 ± 0 n
5	0	9 ± 3 q	3 ± 0 o	0 ± 0 q	0 ± 0 o
5	1	35 ± 1 p	0 ± 0 p	0 ± 0 q	0 ± 0 o
5	3	110 ± 3.5 i	0 ± 0 p	0 ± 0 q	0 ± 0 o
5	5	140 ± 4.5 f	10 ± 0.5 l	0 ± 0 q	0 ± 0 o
5	7	180 ± 5.5 d	5.5 ± 0 n	5 ± 0 pq	0 ± 0 o

[a] Average number of embryogenic calli (15 days) least significant difference (LSD) = 5.7565; average number of embryogenic calli (33 days) LSD = 12.374; average number of plants developed (15 days) LSD = 0.7495; average number of plants developed (33 days) LSD = 0.5119.
[b] Means with the same letter are not significantly different.

formation was observed for immature embryos able to germinate and produce plantlets (Tables 1 and 2; Figure 2A–C).

Irrespective of the explants, 2,4-D is the most widely used growth regulator for wheat callus induction and maintenance (Barro et al., 1998; Arzani et al., 1999). Somatic embryos form on the nutrient medium with a reduced 2,4-D concentration or after a prolonged culture period on the same medium. Osmotic stresses have been shown to be important for induction of embryogenic cultures in wheat (Benkirane et al., 2000). Calluses derived from both treatments (15 and 33 days) displayed an earlier regeneration when treated with 2.0 or $3.0\,mg\,l^{-1}$ 2,4-D mixed with 5.0 and $7.0\,mg\,l^{-1}$ NaCl (Tables 1 and 2) in durum wheat and earlier root formation for the highest concentration of NaCl with bread wheat (data not shown). We obtained the highest number of embryogenic calluses formed with durum wheat when we used 33-day-old calli and $2.0\,mg\,l^{-1}$ of 2,4-D in combination with $7.0\,mg\,l^{-1}$ of NaCl. With MPB-Bobwhite 26, we obtained at 33 days, the maximum number of embryogenic cultures with $1.0\,mg\,l^{-1}$ 2,4-D combined with $7.0\,mg\,l^{-1}$ NaCl (15-day-old calluses) or $1.0\,mg\,l^{-1}$ 2,4-D combined with $1.0\,mg\,l^{-1}$ NaCl (33-day-old calluses). The maximum number of plants obtained with the highly regenerative MPB-Bobwhite 26 was observed in the controls.

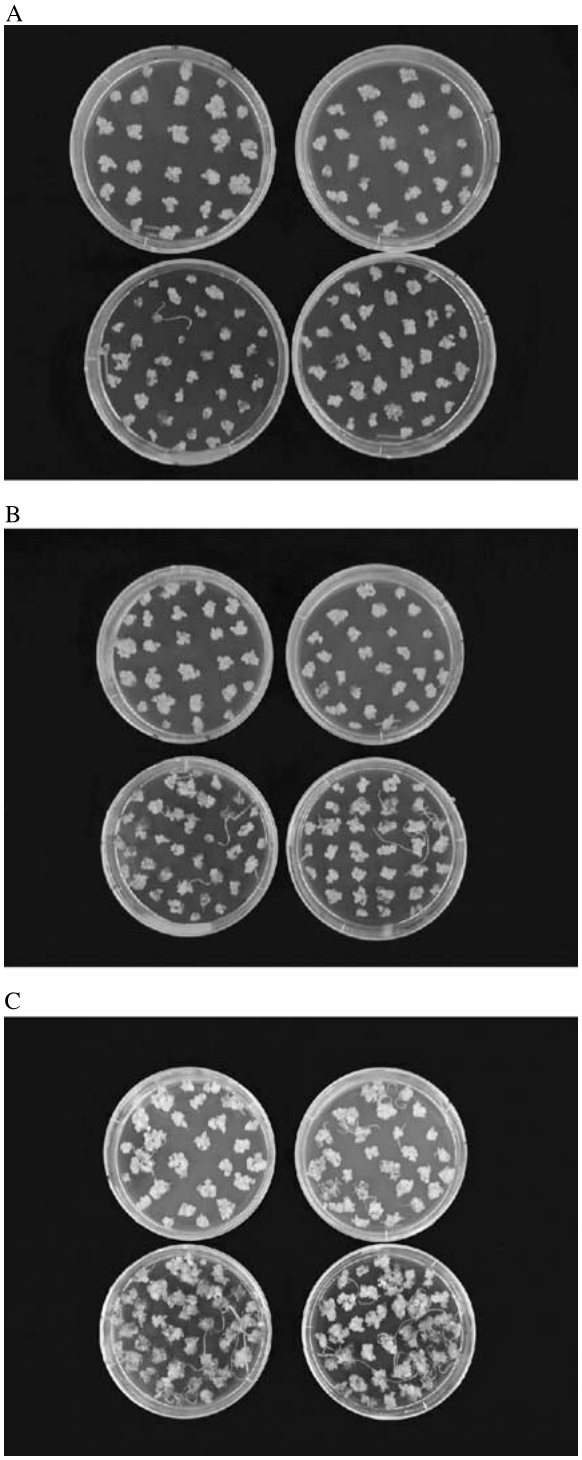

Figure 2. (A) Effect of NaCl and 2,4-D on induction of somatic embryogenesis and plant regeneration in durum wheat cv. Mexicali. On top, 15-day-old, immature embryos from unstressed plants treated with 2.5 mg l^{-1} of 2,4-D (control). On the bottom, identical immature embryos treated with 1 mg l^{-1} of 2,4-D and 3 mg l^{-1} of NaCl. Same Petri dishes after 1 week (B) and 2 weeks (C), showing clear differences in regeneration and plantlet development.

Statistical analyses

The main effects and interactions for 'Mexicali' and MPB-Bobwhite 26, including their response variables, average number of embryogenic calluses, and average number of plants developed, were highly significant at the 0.01 probability level. In general, for the average number of embryogenic calluses, the 'Mexicali' line showed higher values than MPB-Bobwhite 26 (Tables 1 and 2).

Improved induction of embryogenic cultures, has been described based on treating immature embryos with NaCl, for 20 days, prior to transfer to tissue culture medium. We are currently investigating the effects of the NaCl treatment at the physiological and molecular level to better understand the mechanisms that trigger somatic embryogenesis in durum wheat.

Acknowledgements

The authors wish to thank the CIMMYT Applied Biotechnology Center greenhouse team for their technical support. This research was funded in part by the Australian Cooperative Research Center for Molecular Plant Breeding, of which CIMMYT is a core participant.

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