

Effect of 2,4-dichlorophenoxyacetic acid on callus induction and plant regeneration in anther culture of wheat (*Triticum aestivum* L.)

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Abstract Anthers from a doubled-haploid line of spring wheat (*Triticum aestivum* L.) cv. Pavon 76 were plated in liquid P-4 medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) at four concentrations (0.5, 1.0, 2.0, 4.0 mg/l) for 5, 10, 15, and 25 days before being transferred to another medium with the same or reduced 2,4-D concentrations for the remainder of the induction phase for a total of 45 days. Incubation with 0.5 mg/l 2,4-D for 45 days produced lower callus yield and plant regeneration, indicative of insufficient auxin for callus induction. Callus yield and regeneration frequencies were higher with 1.0 mg/l 2,4-D. With 2.0 or 4.0 mg/l 2,4-D, an induction period of 10 or 15 days was sufficient for initiation of callus development. The extended presence of 2–4 mg/l 2,4-D in the medium beyond the initiation phase was detrimental to plant regeneration. Thus optimal callus induction and plant regeneration could be obtained through manipulating the 2,4-D concentration and the duration of its presence in the induction medium.

Key words Androgenesis · Anther culture · Callus induction · Plant regeneration · Wheat · 2,4-dichlorophenoxyacetic acid

Abbreviations 2,4-D 2,4-dichlorophenoxyacetic acid · DH Doubled haploid · DI Phase I induction culture · DII Phase II induction culture · TD Transfer date

Introduction

Doubled-haploid (DH) production through anther culture is a potentially efficient means to generate homozygous true-breeding progeny lines in plant breeding programs. However, there are important impediments to overcome before the full potential of this technology can be realized. With wheat, particularly, the yields of calli, green plants and spontaneous DHs can often be too low for routine use in a breeding program. Although these three aspects of the anther culture response have been shown to be controlled by genotypic factors (Charmet and Bernard 1984; Ekiz and Konzak 1991; Jones and Petolino 1987; Larsen et al. 1991; Lazar et al. 1984; Quimio and Zapata 1990), environmental conditions for growth of donor plants and culture medium composition also have significant effects (Bjornstad et al. 1989; Jones and Petolino 1987; Ouyang 1986). In particular, auxin in the culture medium is considered to be critical for the culture response of wheat anthers and microspores (Ball et al. 1993; Kasha et al. 1989; Liang et al. 1987; Ouyang 1986).

The wide range in callus yields, the high proportions of albino plants regenerated from the calli, and the low frequencies of DHs among green plants recovered seem to be associated with the auxin type, concentration and duration of the induction period (Ball et al. 1993; Kasha et al. 1989; Zheng 1994). However, little systematic work has been undertaken to understand the effect of auxin on androgenesis in wheat anther or isolated microspore culture. Among various types of auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) is employed in most anther/microspore culture systems. Although 2,4-D has been found to elicit rapid cell proliferation and callus formation, the reduction or removal of 2,4-D from the regeneration medium is essential for plant development from calli (Ball et al. 1993; Kasha et al. 1989; Liang et al. 1987; Marsolais and Kasha 1985). In contrast, it has also been reported that auxin-free

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medium produced very high numbers of calli in both *Hordeum vulgare* (Cai et al. 1992) and *Brassica napus* (Polsoni et al. 1988), but the quality of these calli and their regeneration ability were poor. Unlike these two species, wheat anthers produce very few, if any, calli without exogenous auxin in the culture medium. Furthermore, most of the calli, if induced under inadequate auxin concentrations, cannot regenerate into plants. Since 2,4-D is widely used in anther and microspore cultures of wheat, the present study was designed to gain a better understanding of the impact of 2,4-D concentration and treatment duration on callus yields and frequencies of plant regeneration.

Materials and methods

A DH line DH 9177 (Zheng 1994) of spring wheat (*Triticum aestivum* L.) cv. Pavon 76, produced by the authors through anther culture was used in all experiments. Anther donor plants were grown as described by Ekiz and Konzak (1991). The primary and three secondary tillers of donor plants were collected when microspores from the anthers in the central region of the spikes were at the mid to late uninucleate stage. For DH 9177, spikes were usually sampled when their tips were level with the ligule of the penultimate leaf. The developmental stage of microspores was always checked by acetocarmine staining of anthers and subsequent light microscopic observation. The sampled spikes were placed in flasks containing tap water and stored in a dark refrigerator at 4 °C for 48 h. The spikes were surface-sterilized twice with 80% (vol/vol) ethanol for 2-min intervals and the enclosed anthers were excised with fine forceps. Anthers were then plated aseptically in liquid P-4 medium (Konzak and Zhou 1991) supplemented with four concentrations of 2,4-D as described below.

In each of four sets of 72 plastic petri dishes (60 × 15 mm) with 4 ml basic P-4 medium (Ouyang 1986) in a dish, 0.5, 1.0, 2.0, or 4.0 mg/l 2,4-D was supplemented. After a random mixing of petri dishes with added medium, 90 anthers were then transferred into each dish. All petri dishes were then sealed with parafilm and incubated in the dark at 28 C for 5, 10, 15, and 25 days (DI=days for initiation, first phase) denoted TD-5, TD-10, TD-15, and TD-25, respectively. At each of the four dates, 18 plates from each of the four initial 2,4-D concentration sets were randomly selected and divided into three groups, with 6 plates in each group (DII=phase II induction culture). Anthers in the first 6 plates were transferred to 6 plates with fresh medium containing the same 2,4-D concentration, while the second group of 6 plates

were transferred to six plates with 0.2 mg/l 2,4-D in the medium and the last group of 6 plates were transferred to 6 plates with no 2,4-D in the medium. All anthers were cultured in these plates for the rest of the 45-day induction phase.

Prior to transfer to fresh medium, the anthers from each petri dish were rinsed gently with 2,4-D-free medium to minimize the carryover of initial 2,4-D. The calli that developed (>1 mm in diameter) in each plate after 45 days of combined DI and DII culture were counted and then were transferred to 100 × 15 mm plates supplemented with 190-2 medium (Ekiz and Konzak 1991) for plant regeneration. 190-2 is a semi-solid medium that consists of mineral and sugar as nutrients, gelrite as gelling agent and no plant growth regulators. The callus yield was the average number of calli produced from 100 cultured anthers. The regeneration plates were illuminated with 180–200 μmol m⁻² s⁻¹ fluorescent lights for a 16-h photoperiod. After 3 weeks, the plant regeneration frequency was calculated as the total number of plants (with both roots and shoot) regenerated from 100 calli. Data were statistically analyzed first through ANOVA for the main effects of TD, DI, and DII, then with a *t*-test for multiple comparisons among specific 2,4-D combinations.

Results and discussion

Initial medium with 0.5 mg/l 2,4-D

When wheat anthers were incubated in medium with 0.5 mg/l 2,4-D during DI culture, the transfer of anthers to zero or 0.2 mg/l 2,4-D medium for DII culture at all four TDs resulted in lower callus yields when compared to anthers cultured in medium with 0.5 mg/l 2,4-D in both DI and DII. Any reduction of 2,4-D during DII led to a decrease in callus yield ranging from 30 to 50% (Fig. 1). Except for TD-25, the transfer of anthers to 0 or 0.2 mg/l 2,4-D medium also resulted in lower plant regeneration frequencies (Table 1). Within each transfer date, the medium containing 0.5 mg/l 2,4-D during DII culture induced the highest number of calli and plant regeneration frequencies (Fig. 1, Table 1), whereas transfer to zero 2,4-D in DII resulted in the lowest values for both. The longer anthers were incubated in the initial medium, the higher the callus yields and plant regeneration frequencies. A 2,4-D concentration of 0.5 mg/l is inadequate for the optimal induction of callus from wheat anthers.

Table 1 Plant regeneration frequencies per 100 calli induced from media with various concentrations of 2,4-D in DI before the transfer to DII culture. Different letters denote statistical significance (*P*<0.05) within an initial 2,4-D concentration and the same transfer date

2,4-D (mg/l) in DI	2,4-D (mg/l) in DII	Days before the transfer to DII medium			
		5	10	15	25
0.5	0	26.3 c	35.7 bc	38.3 c	46.7 abc
	0.2	35.7 b	40.0 b	47.8 ab	47.0 ab
	0.5	49.0 a	50.5 a	48.3 a	52.3 a
1.0	0	32.5 c	49.3 c	44.5 c	63.4 bc
	0.2	55.7 b	62.7 a	67.6 a	74.8 a
	1.0	63.1 a	60.8 ab	62.7 ab	64.6 b
2.0	0	34.2 c	51.7 b	63.4 ab	51.5 abc
	0.2	53.3 ab	64.8 a	67.6 a	56.2 ab
	2.0	58.8 a	57.4 ab	57.3 b	57.6 a
4.0	0	35.2 b	38.7 b	40.5ab	34.2 abc
	0.2	65.1 a	68.0 a	48.0 a	39.1 a
	4.0	33.7 bc	35.4 bc	33.6 b	37.6 ab