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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

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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

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

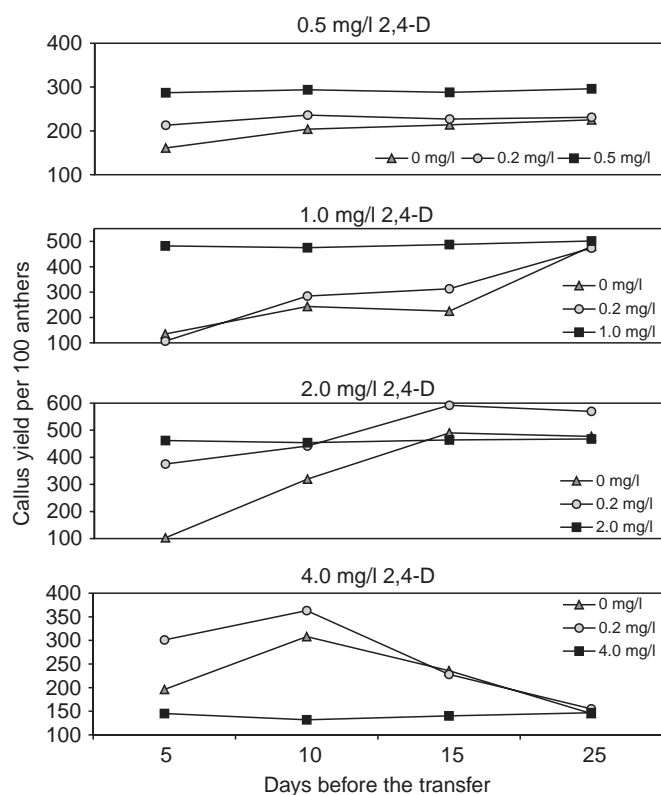


Fig. 1 Average number of calli produced from 100 anthers, cultured in induction medium with 0.5, 1.0, 2.0, and 4.0 mg/l 2,4-D, for 5, 10, 15, 25 days before the transfer to phase II induction media containing 0, 0.2 mg/l or the same 2,4-D as of phase I medium. Calli were counted after 45 days in culture. Numbers were pooled means of 8 replicates for each variable

Initial medium with 1.0 mg/l 2,4-D

When anthers were cultured in medium with 1.0 mg/l 2,4-D, they produced higher callus yields and plant regeneration frequencies compared to anthers cultured in medium with 0.5 mg/l 2,4-D at each of the transfer dates (Fig. 1, Table 1). With 1.0 mg/l 2,4-D in DI culture, the transfer made at TD-5 to medium containing zero or 0.2 mg/l 2,4-D produced significantly fewer ($P < 0.01$) calli than at TD-10, TD-15, and TD-25. No significant difference in callus yields was observed among transfers made at TD-10 and TD-15 to medium containing zero 2,4-D. However, a significant increase ($P < 0.05$) in callus yield was observed when the transfer was made at TD-25 to zero or 0.2 mg/l 2,4-D medium, compared to transfers made at TD-5, TD-10, and TD-15. No significant difference was observed among three media in DII culture when the transfer occurred at TD-25 (Fig. 1). These results suggest that the culture of anthers in medium with 1.0 mg/l 2,4-D for 25 days is sufficient for callus induction. Culture of anthers in such medium for 45 days, a routine procedure (Zheng 1994; Zhou et al. 1991) is not necessary for callus induction.

At TD-5, TD-10, and TD-15, transfers made to zero 2,4-D medium resulted in significantly lower plant regeneration frequencies ($P < 0.05$) than those made to 0.2 or 1.0 mg/l 2,4-D medium. Transfers made at TD-10 and TD-15 to medium containing 0.2 or 1.0 mg/l 2,4-D, and all transfers made at TD-25 yielded virtually the same plant regeneration frequencies (Table 1). It appears that 1.0 mg/l 2,4-D in the induction medium for 10 days may saturate the regeneration capacity of induced calli. However, anthers need to be maintained in the same medium for 25 days to achieve optimum callus yield (Fig. 1). Overall, 1.0 mg/l 2,4-D for 25 days followed by 0.2 mg/l 2,4-D in DII culture is adequate for both optimal callus induction and subsequent plant regeneration (Fig. 1, Table 1).

Initial medium with 2.0 mg/l 2,4-D

With 2.0 mg/l 2,4-D during DI, the transfer made at TD-5 to 0 mg/l 2,4-D medium resulted in low callus yield and regeneration frequency. All transfers made at TD-10, TD-15, and TD-25 to medium with reduced 2,4-D during DII produced the same number of calli as transfers made to medium with 2.0 mg/l 2,4-D. In fact, anthers transferred to medium containing 0.2 mg/l 2,4-D at TD-15 and TD-25 yielded 15–20% more calli than those transferred to medium containing 2.0 mg/l 2,4-D (Fig. 1). Anthers transferred to zero or 0.2 mg/l 2,4-D at TD-10 and TD-15 also produced calli with the same or higher regeneration frequencies than those transferred to 2.0 mg/l 2,4-D (Table 1). Therefore, with 2.0 mg/l 2,4-D in the induction medium, it is crucial to transfer anthers to medium containing less 2,4-D at or before TD-15. In many tissue or cell culture systems, the difficulty in plant regeneration is likely due to continuous culture in medium with a high 2,4-D or auxin level. Our results clearly showed that the continuous presence of a 2,4-D concentration that satisfied callus induction certainly inhibited their further development, hence their subsequent plant regeneration. Once the induction is initiated at 2.0 mg/l 2,4-D, switching the culture to a lower 2,4-D medium is necessary for efficient plant regeneration from calli.

Initial medium with 4.0 mg/l 2,4-D

With 4.0 mg/l 2,4-D in the medium during DI culture, transfers made at TD-5 and TD-10 to medium containing 0 or 0.2 mg/l 2,4-D produced higher callus yields than all other transfers made at these or the other two TDs. Exposure of anthers to 4.0 mg/l 2,4-D beyond TD-10 led to a reduction in callus yield ($P < 0.01$) by as much as 25% (Fig. 1). Anthers that were transferred to 0.2 mg/l medium at TD-5 and TD-10 also produced calli with significantly higher regeneration frequencies than those to other media or at TD-15 and TD-25 (Table 1). Compared to results from 1.0

and 2.0 mg/l 2,4-D during DI, 4.0 mg/l 2,4-D is apparently too high for the induction of calli, even when applied for only 10 days.

When all the data on callus yields and plant regeneration frequencies were analyzed statistically, the ANOVA results showed that the main effects of TD, DI, and DII on callus yields and plant regeneration frequencies were highly significant (Table 2). To gain a better understanding of the effect of specific 2,4-D treatments, differences of least-square means were compared among media with four different initial 2,4-D concentrations as listed in Table 1.

It has been common practice to culture anthers on induction medium with 1.0–8.0 mg/l 2,4-D for at least 45 days before the transfer of calli/embryoids for plant regeneration. However, it is known that the first cell division during induction occurs within 2 days after inoculation (Reynolds and Kitto 1992; Shimada 1989), and the pro-embryoids form by approximately 10 days after culture initiation. Our results showed that 1.0 mg/l 2,4-D in DI and DII, or 2.0 mg/l 2,4-D for 10–15 days followed by a reduced concentration of 2,4-D (0.2 mg/l) are both sufficient for optimal induction of calli. However, calli induced in the first regime had a lower plant regeneration frequency than in the second system. Apparently, if a high concentration of 2,4-D is needed to initiate cell division, a much lower concentration is sufficient for continuing the development of regenerable calli. The reduction of 2,4-D concentration upon completion of the induction phase may be essential for optimal plant regeneration, since new gene products are needed for the transition from callus and/or embryoid development to plant regeneration. These products may only be synthesized when exogenous auxin (2,4-D) is greatly reduced or removed from the culture system (Michalczuk et al. 1992a, b; Zimmerman 1993). The continuous presence of 2–4 mg/l 2,4-D during induction beyond a critical point at which genes encoding products for plant regeneration are expressed is detrimental to the normal development of calli and may cause the loss of their regeneration capacity.

Conclusion

These studies have shown that the threshold level of 2,4-D for callus induction from wheat anthers seems to

Table 2 ANOVA showing the effect of main factors (TD, DI, DII) on callus yields and plant regeneration frequencies

Source	DF	Callus yield Pr>F	Regeneration frequencies Pr>F
TD	3	0.0001	0.0004
DI	3	0.0001	0.0001
DII	5	0.0001	0.0023
TD × DI	9	0.0001	0.0001
TD × DII	15	0.0001	0.0017

be 1.0–2.0 mg/l. Within this range, a lower 2,4-D concentration can be compensated by a longer induction period, or a higher 2,4-D concentration by a shorter period of induction meeting the requirement for auxin to satisfy the induction of calli from cultured anthers. Optimal callus induction and plant regeneration can be obtained when anthers are incubated in a medium with 2.0 mg/l 2,4-D for less than 15 days before switching to 0.2 mg/l 2,4-D. Also effective is first culturing anthers in medium with 1.0 mg/l 2,4-D for 25 days and then transfer to a 0.2 mg/l 2,4-D medium. The availability of an optimized anther culture system for DH production could greatly enhance basic research in wheat genetics and accelerate wheat breeding programs.

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