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Normalization of the DNA content of telophase cells from wheat calli by nutrient modifications

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Summary. Cytophotometric analyses were conducted to determine whether the DNA content of wheat callus varied by tissue culture medium or age of callus. Wheat, *Triticum aestivum* L. line PCYT-20, was cultured on three variations of the Murashige and Skoog (1962) growth medium. At the end of 2, 4, 6 and 8 weeks, samples were collected and prepared for Feulgen cytophotometry. Standards for the DNA measurements were readings from 100 telophase nuclei in wheat meristematic root tips. Amounts of DNA per nucleus present in telophase cells from callus grown on single-strength MS indicated that ploidy level increased 52%, 74% and 39%, respectively, over time from 2, 4, and 6 weeks as compared to the double-strength MS medium, and 29%, 60% and 32%, respectively, when coconut water was added to the single-strength MS culture medium. The shape of the mitotically-active cells in callus was more variable than in root tips cells. Callus grown on double-strength MS medium produced more shoots than callus grown on single-strength MS. Double-strength MS medium and, to a lesser extent, additional sucrose and organic nitrogen overcame the effects of 2,4-D on DNA amplification. Improved media may reduce the somaclonal variation induced by tissue culture.

Key words: DNA amplification – Tissue culture – Cytophotometry

Introduction

Nuclear DNA amplification in many annual angiosperms occurs during cell growth and differentiation or in response to environmental conditions (Bennett and Smith 1976; Cullis and Goldsborough 1980; Flavell 1975; Olszewska et al. 1988; Price 1976; Walbot and Cullis 1985). In plant cell tissue cultures, certain plant growth regulators (PGR's), especially 2,4-dichlorophenoxyacetic acid (2,4-D), may induce mitotic aberrations, endomitoses, amplifications, deletions, translocations or endoreplications (Chen and Chen 1980; Ghosh and Gadgil 1979; Walbot and Cullis 1985). The mechanisms underlying these variations have been extensively reviewed (Bayliss 1980; Evans 1986; Larkin and Scowcroft 1981; Larkin et al. 1984; Scowcroft et al. 1985).

Response to PGR's may result in rapid genomic changes that give rise to variation in the nuclear DNA content. Dolezel and Novak (1985) observed an accumulation of *Allium sativum* L. cells with nuclear DNA content as high as 64C, depending on the hormone treatment. They attributed the accumulation to uninterrupted DNA synthesis and/or an endogenous pool of DNA precursors. Also, quantitative and qualitative heritable changes have been observed in the nuclear DNA in dihaploid plants of *Nicotiana sylvestris* L. that were derived by consecutive cycles of androgenesis (DePaepe et al. 1982). Rapid DNA variation leading to heritable changes have likewise been observed in flax, *Linum usitatissimum* L., varieties that were grown for a single generation in tissue culture or different, characterized stress environments (Walbot and Cullis 1985 and references therein). Although not fully documented, embryogenesis in several plant cell cultures is consistent with DNA amplification and deamplification (Jackson 1980; Nabors et al 1980; Walbot and Cullis 1985). In contrast, Karlsson and Vasil (1986) reported that embryogenic cell suspension cutures of *Panicum maximum* Jacq. and *Pennisetum purpureum* Schum. maintained in Murashige and Skoog's (1962) (MS) medium containing coconut water and 2,4-D

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were predominantly normal cells with 2C DNA values. Other researchers (Bennett and Smith 1976; Bennett et al. 1982; Walbot and Cullis 1985) have observed that while DNA C values can vary within as well as between plant species, the variation is usually less within than between species. The objectives of this study were to evaluate the effects of three different media on the genetic stability of calli induced from 10- to 12-day-old immature wheat embryos by comparing their DNA content with that of normal somatic cells from root tips. The effects of these media on shoot production and plantlet regeneration were also analyzed.

Materials and methods

Plant material

Spring wheat, *Triticum aestivum* L. line PCYT-20, was grown in a Kidman sandy loam (Calcic haploxerolls):peat moss:vermiculite (3:1:1) in 150 × 165 mm plastic pots under greenhouse conditions with a temperature regime of 25°/18 °C day/night. Lighting from high-pressure sodium lamps (1,000 watts) provided a 16/8 h day/night regime with an irradiance of 500 $W m^{-2}$ (400–700 nm). Ten to twelve days after anthesis young embryos were excised and randomly assigned to the media of Maddock et al. (1983), Ozias-Akins and Vasil (1983) and Sears and Deckard (1983) (Table 1). These media are referred to hereafter as modified MMS-CW (CW = coconut water), DMS, and MMS, respectively. The culture procedure followed that of Carman et al. (1988).

DNA cytophotometry

After 2, 4, 6 and 8 weeks of uninterrupted growth, calli were sampled and prepared for Feulgen cytophotometry (Berlyn and Miksche 1976). Simultaneous processing was not possible because a large number of preparations had to be analyszed. Variations in stain batches and DNA quantity in Feulgen units between slides and varying preparation times were present. Thus, squashes from wheat meristematic root tips (average 2C DNA amount per diploid genome was 11.5 pg) were concurrently stained for each group of slides and used as an internal standard in order to make all results comparable and to convert relative Feulgen units into picograms of DNA (Bennett and Smith 1976). Feulgen/DNA absorptions were measured at random on 100 individual early telophase cell nuclei of each treatment at wavelengths of 508 and 550 nm using a Zeiss MP-01 microspectrophotometer.

Growth measurements and data analysis

After 18 weeks without new culture or transfer, calli from different media were evaluated for number of shoots, roots and the production of plantlets. Data were analyzed in a randomized complete block design.

Results

The nuclear DNA content of callus cells induced from immature wheat embryos increased when grown on MMS or MMS-CW at 2 and 4 weeks, but decreased at 6 and 8 weeks when compared to the DMS treatment (Table 2). At 8 weeks all treatments approached the DNA level of wheat root tips (average 34.7 pg/nucleus). The nuclear DNA content increased by 29%, 60% and 35% on MMS-CW medium and by 65%, 74% and 39% on MMS medium after 2, 4 and 6 weeks, respectively, compared to comparable values induced by the DMS medium. The nuclear DNA content of callus cells grown on DMS was not significantly different ($P < 0.10$) from that observed in wheat root tips.

After 18 weeks without subculturing, significantly ($P < 0.10$) more plantlets and shoots formed on DMS and significantly ($P < 0.10$) more roots formed on MMS (Table 3). In a large-scale survey of ten wheat lines, there were twice as many somatic embryos per callus on DMS than on MMS or MMS-CW (Carman et al. 1988). The improved genetic stability of cultures incubated on DMS may have improved morphogenesis. This medium may also reduce somaclonal variation caused by mutations or chromosomal aberrations.

Table 1. Components of three media used for induction of embryogenic callus from immature wheat embryos

Component	Medium[a]		
	DMS	MMS	MMS-CW
MS inorganic salts	2×	1×	1×
Concentrations (m*M*)			
Myo-inositol	0.56		0.56
L-asparagine		1.0	
Sucrose	58.43	58.43	87.64
Concentrations (*μM*)			
Nicotinic acid	4.06		4.06
Pyridoxine · HCl	2.43		2.43
Thiamine · HCl	0.30	1.50	0.30
2,4-D	4.52	4.52	4.52
Concentrations (mg/l)			
Casein hydrolysate	100		
Difco bacto agar	10,000	7,000	6,000
Coconut water (V/V)			10%
pH	5.75	5.80	5.80

[a] DMS, Ozias-Akins and Vasil 1983; MMS, Sears and Deckard 1982; MMS-CW, Maddock et al. 1983

Table 2. Nuclear DNA content of calli induced from immature wheat embryos on three different media

Medium[a]	DNA per nucleus (pg)			
	2 weeks	4 weeks	6 weeks	8 weeks
	x ± SE	x ± SE	x ± SE	x ± SE
DMS	33.3 ± 1.3	29.7 ± 3.1	31.5 ± 1.7	36.6 ± 2.2
MMS	96.9 ± 3.4	112.8 ± 2.4	51.3 ± 2.1	41.4 ± 2.6
MMS-CW	46.8 ± 4.6	74.1 ± 2.8	48.3 ± 1.7	39.6 ± 2.5

[a] See footnotes of Table 1

Table 3. Plantlets, shoots and roots (means ± standard errors) produced per immature embryo incubated on DMS, MMS-CW and MMS after 18 weeks without subculture

Variable	Medium [a]		
	DMS	MMS	MMS-CW
		per callus	
	$\bar{x} \pm SE$	$\bar{x} \pm SE$	$\bar{x} \pm SE$
Plantlets	11.4 ± 2.4	3.6 ± 0.7	8.2 ± 1.0
Shoots	3.8 ± 0.9	2.0 ± 0.8	2.0 ± 0.3
Roots	3.5 ± 0.3	9.4 ± 1.2	3.6 ± 0.7

[a] See footnotes of Table 1

Discussion

A comparison of the constituents of the three media (Table 1) suggests several possible causes of the observed variation in DNA content. The MMS medium included 1.0 mmol l^{-1} L-asparagine but no myo-inositol, nicotinic acid or pyridoxine HCl, while DMS contained twice the amount of inorganic salts, 100 mg l^{-1} casein hydrolysate and more agar. The lower agar concentration in MMS probably had little effect on DNA content because there was an intermediate increase on MMS-CW, which had even a lower agar concentration than MMS. The total amino acid content of casein hydrolysate (approximately 2 to 3 mmol l^{-1} in glycine equivalents) in DMS was two- to three-fold higher than the amount of L-asparagine present in MMS, and the mixture of amino acids in the former medium may have enhanced cellular utilization of nitrogen. The two-fold increase in organic salts and the two- to three-fold increase in organic nitrogen in DMS and the lack of myo-inositol and other vitamins in MMS appear to be the most likely reasons why the DNA content of calli grown on these two media differed dramatically.

The MMS-CW medium also contained 3% sucrose (2% for DMS and MMS) and 10% coconut water. Large amounts of free amino acids in coconut water may have elevated the amino acid concentration of MMS-CW to as high as 10 mmol l^{-1} in glycine equivalents. Thus, the high nitrogen concentration of DMS (123 mmol l^{-1}, organic and inorganic forms) may explain or account for the minimum variation in DNA content. These results are consistent with those of Carman et al. (1988) who found that DMS was significantly better than either MMS or MMS-CW in embryogenic callus induction.

Other workers have documented that 2,4-D increases the DNA content of callus cultures, which they attributed to uninterrupted DNA synthesis (Gosh and Gadgil 1979; Dolezel and Novak 1985; Walbot and Cullis 1985). In the present study, the levels of nuclear DNA at 8 weeks in mitotically active callus cells grown on MMS and MMS-CW returned to the average DNA levels observed in root tips (Table 2). This "return" to normalcy has been shown to correspond with the degradation of 2,4-D by wheat tissues (Bristol et al. 1977).

The fact that the DNA content of callus cells incubated on MMS-CW increased 2 weeks later than in the MMS may be attributed to the additional energy provided by the high level of sucrose. At 4 weeks, however, the sucrose may have been depleted, allowing the effect of 2,4-D to be expressed. On MMS medium, which lacked additional sucrose, extra inorganic salts, and extra organic nitrogen, the effect of 2,4-D was expressed as early as 2 weeks. After 6 and 8 weeks, nearly all of the 2,4-D may have been metabolized by the cells, allowing the nuclear DNA content to approach that observed in wheat root tips. These data disagree with those of Carlberg et al. (1984) who reported that *Solanum tuberosum* L. nuclei with high DNA-content were found early in the culture regimes and the frequency of these nuclei increased with time in culture.

The effect of sucrose concentration on embryo growth and germination is well documented (Narayanaswami and Norstog 1964). Raghavan and Torrey (1963) demonstrated that globular *Capsella* proembryos as small as 81 μm could be grown on media containing relatively low sucrose concentrations (2%) if the media also supplied kinetin, adenine and IAA. They also noted that either increasing the concentration of sucrose to 12–18% or increasing the level of macronutrient salts had similar effects. No somatic embryos were formed from zygotic embryos of sunflower (*Helianthus annuus* L.) when 3% sucrose was used in combination with IAA, NAA, picloram, dicamba or 2,4-D at 0.1, 0.5, 1, 5 or 10 mg l^{-1} (Finer 1987). However, with high levels of sucrose (6% and 12%) in the media, 3.3 mg l^{-1} dicamba or 1 mg l^{-1} 2,4-D was optimum for somatic embryo initiation. Our data reflect their results.

The plant sap surrounding wheat embryos (cavity sap) contains 10–50 m*M* sucrose, small amounts of hexoses and high concentrations of oligosaccharides (Ho and Gifford 1984). These carbohydrates are essential for growth rate and size of kernels. In vitro sucrose supplies energy to actively dividing cells. The amount of sucrose in DMS and MMS (58.43 m*M*) is approximately equal to that found in the cavity sap.

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