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Cytoplasmic effects on the tissue culture response of wheat (*Triticum aestivum*) callus

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Summary. Calli were initiated from immature embryos of eight lines of hexaploid wheat (*Triticum aestivum* L. em. Thell) with different cytoplasms, the euplasmic nuclear donor 'Chinese Spring' and seven alloplasmic lines derived from wild relative species of the genera *Triticum* and *Aegilops*. The calli were found to differ in their initial growth rates, their sensitivity to 2,4-D and their ability to organise shoot primordia, demonstrating that the cytoplasm can significantly affect the behaviour of tissues in culture. The potential for improving the responses of tissues in culture by cytoplasmic changes is noted.

Key words: Cytoplasm – Wheat – Tissue culture – Callus

Introduction

The repeated backcrossing of hexaploid wheat (*Triticum aestivum* L. em. Thell) as pollen parent, onto related wild or cultivated species of the genera *Triticum* and *Aegilops*, results in the establishment of alloplasmic lines in which the wheat nuclear genome is substituted into an alien cytoplasm.

Modifications to phenotypic characters have been observed in alloplasmic wheat plants and include pistilloidy (Kihara 1951), the production of haploids and twin seedlings (Kihara and Tsunewaki 1962), plant height, flowering date, dry matter production and various components of yield (Maan 1979). The most important and most studied effect associated with the substitution of a nucleus into an alien cytoplasm is male sterility (Tsunewaki 1982). In wheat and maize (*Zea mays*) cytoplasmic male sterility is controlled by the interaction of one or two nuclear genes and a number of cytoplasmic genes (Gracen and Grogen 1974; Tsunewaki 1982). It is likely that some of the other observed effects of alloplasmy will also prove to be the result of nuclear-cytoplasmic gene interactions. Shimada (1978) demonstrated that regenerable callus could be established from the immature embryos of wheat (cvs. 'Chinese Spring' and 'Salmon'). Recently there have been reports of significant differences in the regeneration of calli from immature embryos of breeders lines and commercial varieties of spring and winter wheats (Sears and Deckard 1982; Maddock et al. 1983).

In this paper we report the variation in behaviour in tissue culture of calli derived from immature embryos of eight cytoplasmic lines of hexaploid wheat.

Materials and methods

Plant material

The lines used in these experiments were a euplasmic line of the nuclear donor 'Chinese Spring' (CS) and seven alloplasmic lines having the CS nucleus substituted into the cytoplasm of *Aegilops bicornis* ((*bicornis*)-CS), *Aegilops ovata* ((*ovata*)-CS), *Aegilops squarrosa* ((*squarrosa*)-CS), *Triticum timopheevi* ((*timopheevi*)-CS), *Aegilops uniaristata* ((*uniaristata*)-CS), *Aegilops umbellulata* ((*umbellulata*)-CS) and *Triticum zhukovskyi* ((*zhukovskyi*)-CS). These lines were produced by Dr. S. S. Maan, USA and are maintained at the Plant Breeding Institute, Cambridge. All the lines have had more than eight backcrosses.

Seeds were germinated on water soaked filter papers in Petri dishes at 20 °C in the dark. The seedlings were potted into John Innes No. 1 compost in "Jiffy-pots" and vernalised for four weeks at 4 °C. Plants were then potted into 4″ pots and grown to maturity in a greenhouse at 20 °C±3 °C, under 16 h daylength. Individual ears were bagged at ear emergence and allowed to self-pollinate. Ears of (*timopheevi*)-CS and (*uniaristata*)-CS were completely male sterile, so that ears of these lines were fertilised with CS pollen.

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Tissue culture

Fourteen days after anthesis the immature seeds from the middle 8–10 florets of each ear were harvested. Harvested grains were immediately surface sterilised in 10% Domestos (Lever Bros., U.K.) for 10 min, followed by two sterile distilled water washes. The immature embryos were excised under a dissecting microscope and placed on agar medium with the scutellum exposed and the hypocotyl/radicle embedded in the medium. The media used were those specified by Sears and Deckard (1982). The basal medium contained the inorganic salts of Murashige and Skoog (1962), supplemented with 150 mg/l L-asparagine, 0.5 mg/l thiamine, 20 g/l sucrose and 7 g/l agar. The pH was adjusted to 5.8 before autoclaving. Callus from 14-day old embryos was initiated on the basal medium containing 1.0 mg/l 2,4-D. One month after inoculation the number of calli formed on the initiation medium and the number of calli developing green spots were recorded. The maximum length and the maximum width at right angles to the longest axis were measured for each callus, and the area of an elipse with these dimensions was calculated as an approximate index of callus growth. One month old calli were transferred to maintenance medium (basal medium with 0.5 mg/l 2,4-D). After one month on maintenance medium the number of calli with green spots and shoots was recorded and the calli transferred to regeneration medium (basal medium with 0.1 mg/l 2,4-D). After one month on regeneration medium the number of calli with shoots was recorded and regenerating calli classified into groups having 1–3, 4–6, 7–9 and 10+shoots per callus. Only shoots which exceeded 1 cm in length were recorded.

All cultures were maintained at 27 °C±1 °C.

Approximately two-thirds of the calli initiated from each line were cultured under continuous illumination, the remainder were cultured in the dark.

Results

In all the tested lines 14 day old embryos excised from immature grains and cultured under the described conditions produced calli with high efficiency. Callus growth from embryo/scutullar tissue became visible during the first week in culture and there were no obvious differences in the time of appearance of callus. When embryos were cultured in the light there was significant variation among the lines in their efficiency in initiating callus. All the embryos of (*ovata*)-CS, (*timopheevi*)-CS, (*umbellulata*)-CS and (*uniaristata*)-CS produced calli on initiation medium, the other lines were less efficient, but only embryos of CS and (*squarrosa*)-CS were significantly less efficient at forming callus than the most efficient lines (Table 1).

There was no significant variation in callus initiation among embryos cultured in the dark although the non-significant interaction item in the overall analysis suggests that a similar pattern of variation between lines occurred in both the light and the dark. Light had no significant effect on the frequency of callus initiation.

The callus produced from different lines was of similar appearance with a loose friable texture and pale yellow-white colouration.

Table 1. Callus induction (% of embryos plated which produce callus on initiation medium)

Cytoplasm	Light	*n*	Dark	*n*
aestivum	91 a	65	100	30
bicornis	98	63	100	30
ovata	100 a	60	97	30
squarrosa	94 b	66	100	30
timopheevi	100	30	–	
unbellulata	100 ab	62	100	30
uniaristata	100 a	60	100	30
zhukovskyi	97	60	97	34

Cytoplasms followed by a particular letter are singificantly different from the first cytoplasm in the list with that letter (Chi^2 analysis of induction frequencies)

n = number of calli in sample

Chi^2 analysis of callus induction frequencies of seven cytoplasmic lines grown under light and dark conditions

Light v Dark	df 1	2.64
Cytoplasms	df. 6	14.02*
Interaction	df. 6	10.69
Total	df. 13	27.35**

The initial growth rate of calli, measured as callus area on a per petri dish basis, showed significant variation between the growth rates of calli in the dark and light. Dark grown calli of all lines were significantly larger than those grown in the light. There were significant differences in callus size between lines both in the light and the dark. In the light only (*zhukovskyi*)-CS produced significantly more callus tissue than CS, in the dark both (*bicornis*)-CS and (*zhukovskyi*)-CS were significantly different from CS (Table 2).

A proportion of the calli from each line developed green spots, regarded as indicative of potential morphogenic activity in cereal calli (Green and Phillips 1975; Sears and Deckard 1982), during culture. The lines demonstrated significant variation in the number of calli which produced green spots during the callus initiation phase. All the cytoplasms (except that of *Ae. umbellulata*) when compared with CS showed a significant stimulatory effect on green spot development (Table 3). None of the calli initiated in the dark produced green spots. There were some significant differences between lines for this character (Table 3).

The growth of calli for one month on maintenance medium, in the light, resulted in an increase in the number of calli developing green spots (Table 4). This increase was significant in CS, (*bicornis*)-CS, (*squarrosa*)-CS, (*umbellulata*)-CS and (*zhukovskyi*)-CS. In the dark some (*squarrosa*)-CS and (*umbellulata*)-CS calli produced green spots. There was significant variation between some of the lines as to the number of calli producing green spots in the light. In particular the

Table 2. Initial rate of growth of calli. Average area (in mm²) of callus/petri dish (10 calli/dish) after 1 month on initiation medium

Cytoplasm	*n*	Light mean	*n*	Dark mean
aestivum	11	166 a	5	202 a
bicornis	11	202 b	5	308 ab
ovata	10	117 bc	5	161 bc
squarrosa	11	164 d	5	234 bcd
timopheevi	6	206 ce	–	– –
umbellulata	11	133 bef	5	192 be
uniaristata	10	172 cg	5	213 bf
zhukovskyi	12	239 acdefg	6	297 acedef

Cytoplasms followed by a particular letter are significantly different from the first cytoplasm in the list with that letter (analysis of variance)

n = number of petri dishes in sample

Analysis of variance for the initial growth rate of calli of seven cytoplasmic lines grown under light and dark conditions

Light v dark	df. 1	MS. 2,062,575***
Cytoplasms	df. 6	MS. 785,031***
Interaction	df. 6	MS. 46,453
Error	df. 98	MS. 74,243

Table 3. Morphogenic calli (% of calli producing green spots on initiation medium)

Cytoplasm	Light	*n*	Dark	*n*
aestivum	2 a	65	0	30
bicornis	11 ab	63	0	30
ovata	17 ac	60	0*	30
squarrosa	17 ad	66	0*	30
timopheevi	30 abe	30	–	–
umbellulata	3 cdef	62	0*	29
uniaristata	45 abcdfg	60	0*	30
zhukovskyi	20 afg	60	0*	34

Cytoplasms followed by a particular letter are significantly different from the first cytoplasm in the list with that letter. Rows followed by an asterisk indicate that the light and dark grown calli are singificantly different (Chi² analysis of the frequency of green spot production)

n = number of calli in sample

Chi² analyis of the frequencies of green spot production on calli of seven cytoplasmic lines grown under light and dark conditions on initiation medium

Light v Dark	df. 1	40.86***
Cytoplasms	df. 6	52.32***
Interaction	df. 6	25.01***
Total	df. 13	118.19***

calli of (*bicornis*)-CS, (*squarrosa*)-CS, (*uniaristata*)-CS and (*zhukovskyi*)-CS were significantly more active in this respect than those of CS. In all lines a higher proportion of the calli grown in the light produced green spots than those grown in the dark (Table 4).

Table 4. Morphogenic calli (% of calli producing green spots on maintenance medium)

Cytoplasm	Light	*n*	Dark	*n*
aestivum	23 a	65	0*	30
bicornis	45 a	60	0*	30
ovata	31 c	58	0*	29
squarrosa	50 acd	58	7*	30
timopheevi	38	29	–	
umbellulata	21 bde	62	3*	29
uniaristata	54 abce	46	0*	30
zhukovskyi	45 ae	58	0*	33

Cytoplasms followed by a particular letter are significantly different from the first cytoplasm in the list with that letter. Rows followed by an asterisk indicate that the light and dark grown values are significantly different from another (Chi² analysis of the frequency of green spot production)

n = number of calli in sample

Chi² analysis of the frequencies of green spot production on calli of seven cytoplasmic lines grown under light and dark condition on maintenance medium

Light v Dark	df. 1	91.91***
Cytoplasms	df. 6	18.91**
Interactions	df. 6	19.75**
Total	df. 13	130.57***

Some of the lines grown in the light on maintenance medium regenerated shoots, but there was no significant variation between lines for this character (Table 5). After one month on regeneration medium in the light, all lines had an increased proportion of calli producing shoots. The increase was significant in (*bicornis*)-CS, (*ovata*)-CS, (*squarrosa*)-CS, (*umbellulata*)-CS and (*zhukovskyi*)-CS. In the dark, four lines showed an increase in shoot regeneration, and this is reflected in the significant interaction item of the overall analysis. A significantly greater number of (*bicornis*)-CS, (*ovata*)-CS and (*squarrosa*)-CS calli produced shoots on regeneration medium in the light than in the dark (Table 5). Except in one or two cases, calli regenerated no more than three shoots per callus after one month on regeneration medium, there was no variation between lines.

Cytoplasmic relationships

The wild *Aegilops* and *Triticum* species, some of which were the cytoplasm donors for the alloplasmic lines we have tested, are related both to one another and to CS through common ancestors (Tsunewaki et al. 1976; Ogihara and Tsunewaki 1982). In order to test whether the degree of relatedness of the nuclear and cytoplasmic donors accounts for the observed differences, the lines were grouped according to two classifications and the variation within and between groups was tested by Chi² or variance ratio analysis.

Table 5. Regenerative activity (% calli producing shoots on maintenance and regeneration medium)

Cytoplasm	Maintenance				Regeneration					
	Light	*n*	Dark	*n*	Light	*n*	Dark	*n*	I	II
aestivum	3	65	0	30	17	30	0	15		
bicornis	5	60	0	30	36	33	0	18	*	*
ovata	0	58	0	29	26	43	0	29	*	*
squarrosa	0	58	0	30	30	51	7	30	*	*
timopheevi	0	29	–		12	33	–			
umbellulata	3	62	7	29	21	42	14	29		*
uniaristata	2	46	0	30	11	47	3	30		
Zhukovskyi	3	58	3	33	16	38	17	30	*	*

An asterisk in column I indicates that significantly more calli of that cytoplasm produced shoots in the light than in the dark on regeneration medium. An asterisk in column II indicates that calli of that cytoplasm regenerated more shoots on regeneration than maintenance medium. (Chi^2 analysis of the frequency of shoot regeneration)

n = number of calli in sample

Chi^2 analysis of the frequencies of shoot regeneration on calli of seven cytoplasmic lines grown under light and dark conditions on maintenance medium

Light v Dark	df. 1	0.72
Cytoplasms	df. 6	8.61
Interaction	df. 6	4.92
Total	df. 13	14.25

Chi^2 analysis of the frequencies of shoot regeneration on calli of seven cytoplasmic lines under light and dark conditions on maintenance medium

Light v Dark	df. 1	19.77**
Cytoplasms	df. 6	8.56
Interaction	df. 6	12.75*
Total	df. 13	41.08***

Table 6. Analysis of the variation within and between groups of cytoplasms, classified according to the nuclear ploidy level of the cytoplasm donor

		Callus induction Chi^2	Callus growth MS	Morphogenic calli	
				(initiation) Chi^2	(maintenance) Chi^2
Total	df. 7	19.03***	1,271,510.4 –	58.35***	26.21***
Between groups	df. 2	9.18*	72,005.0 ns	5.48 ns	2.77 ns
Within groups	df. 5	9.85	1,751,312.6 –	52.87***	23.44***

Table 7. Analysis of the variation within and between groups of cytoplasms, classified according to the relatedness of their chloroplast DNAs

		Callus induction Chi^2	Callus growth MS	Morphogenic calli	
				(initiation) Chi^2	(maintenance) Chi^2
Total	df. 7	19.03***	1,271,510.4 –	58.35***	26.21***
Between groups	df. 4	17.28*	55,105.8 ns	54.41***	16.35**
Within groups	df. 3	1.75 ns	2,960,050.0 –	3.94 ns	9.86*

The first classification was according to the ploidy level of the cytoplasmic donor. Thus, (*bicornis*)-CS, (*squarrosa*)-CS, (*umbellulata*)-CS and (*uniaristata*)-CS are diploids, (*ovata*)-CS and (*timopheevi*)-CS are tetraploids and CS and (*zhukovskyi*)-CS are hexaploids.

The second classification was based on that of Ogihara and Tsunewaki (1982). Lines were grouped together which had chloroplast DNA restriction patterns that differed by less than three fragments. On this basis (*aestivum*)-CS, (*squarrosa*)-CS, and (*uniaristata*)-CS are separate groups, (*timopheevi*)-CS, (*zhukovskyi*)-CS and (*ovata*)-CS are a group and (*bicornis*)-CS and (*umbellulata*)-CS are a group.

When lines were classified according to the ploidy level of the cytoplasmic donor there was significant variation both within and between groups (Table 6). The classification based on cytoplasmic relationship in most cases eliminated significant variation within the groups (Table 7). This supports the hypothesis that the observed effects are determined by the cytoplasm and/or nuclear-cytoplasmic interactions and that the pattern of these effects reflects the degree of relatedness of the cytoplasms.

Discussion

The cytoplasmic background of calli initiated from immature embryos significantly affects their responses in tissue culture. Sears and Deckard (1982), using similar culture conditions, reported a wide range of callus initiation frequencies for different wheat genotypes. Under our conditions the euplasmic nuclear donor CS is equivalent to their best lines, initiating calli with high efficiency. In contrast to the reported genotypic effects on callus initiation the alien cytoplasms have comparatively small effects on the efficiency of callus induction. Significantly, the differences observed all result from increases in the efficiency of callus initiation in cytoplasmic lines over CS. The area of callus produced, when dealt with on a per petri dish basis, is a good estimate of callus growth (Mathias, unpublished data). All the lines produced more callus under dark growth conditions than light and in all cases the differences were proportionately equal. Clearly there was a general inhibition of callus growth in the light; specific interactions between cytoplasms and light or dark growth conditions that affected growth were not found.

As the initiation, maintenance and regeneration media differ only in the amount of 2,4-D they contain, responses of calli to particular media must be interpreted as responses to a particular concentration, or change in concentration, of 2,4-D. The lines all showed the same general response as calli were transferred from higher to lower levels of 2,4-D. The number of calli developing green spots and shoots increased with reduced concentration, a pattern that agrees with previous reports (Shimada 1978; Sears and Deckard 1982; Maddocks et al. 1983) that high 2,4-D levels inhibit organisation of shoot primordia in cereal calli. The green spots observed on calli resemble, in description and behaviour, those described by Shimada (1978) and Sears and Deckard (1982) as giving rise to leafy shoots.

On initiation medium the proportion of the total CS and (*umbellulata*)-CS calli which produced green spots was significantly smaller than most of the lines indicating that the cytoplasm stimulated callus morphogenesis.

Differences between the cytoplasmic lines and the nuclear donor CS are a measure of the importance of the cytoplasm in modifying the response of the tissues to particular levels of 2,4-D. The responses of some lines to different media were significantly different from one another, as well as CS, suggesting that specific interactions between the CS nucleus and particular cytoplasms may be important in determining tissue culture responses. Some calli of each line produced shoot primordia in the light, on initiation medium. None of the lines produced green spots in the dark, this may be a direct effect of the growth conditions on primordia initiation but may also be due to inhibition of primordia 'greening' – rather than inhibition of primordia formation. There was a significant interaction of cytoplasms with the growth conditions indicating differential responses between cytoplasms.

In most of the lines transfer to maintenance medium significantly increased the number of calli with green spots. There was significant variation between lines and (*bicornis*)-CS, (*squarrosa*)-CS, (*uniaristata*)-CS, (*zhukovskyi*)-CS were significantly more morphogenic than CS. Several lines regenerated shoots from calli cultured in the light on maintenance medium but the differences between lines were not significant. Most lines regenerated significantly more shoots on regeneration than maintenance medium but the regeneration frequencies for lines on regeneration medium were not significantly different. Shoot regeneration was significantly affected by the growth conditions. Clearly the capacity to organise shoot primordia is modified by the cytoplasmic background, but this interaction of the cytoplasm with the 2,4-D concentration of the medium does not extend to the subsequent development and outgrowth of these primordia. None of the tested cytoplasms has a significant effect on the outgrowth of shoots from shoot primordia. In contrast, in the most regenerable lines both 2,4-D concentration and light affected shoot development.

To our knowledge this is the first report, in any species, that the cytoplasm affects the behaviour of tissues in culture. Reported differences in the regeneration activity of calli from a range of genotypes (Sears

and Deckard 1982; Maddock et al. 1983) have indicated that regeneration from tissue culture is controlled by one, or more probably a number of nuclear genes. Our observations make it clear that in some circumstances the cytoplasm also has a significant effect on the behaviour of tissues in culture. The responses may be determined by the cytoplasm alone or result from an interaction of the cytoplasm with the CS nucleus. A number of modified phenotypic characters occurring in cytoplasmic substitution lines have been identified as the results or specific nuclear-cytoplasmic interactions (Kihara 1982). Our observations of modified responses to hormones and changes in culture behaviour may well reflect similar interactions.

We grouped lines according to the relatedness of their cytoplasms, following Ogihara and Tsunewaki's classification (1982) of *Aegilops* and *Triticum* species. The groups formed are largely uniform in their tissue culture responses, supporting the hypothesis that the observed effects are determined by the cytoplasm and/or nuclear-cytoplasmic interactions and that the pattern of these effects reflects the relatedness of the cytoplasms.

It is clear that the cytoplasm can affect the proportion of embryos which produce calli and the proportion of those calli which are morphogenically active. In comparison to CS certain cytoplasms stimulate the formation of shoot primordia but none of those tested affected the subsequent outgrowth of shoots.

In interpreting these experiments it should not be overlooked that the cytoplasm may be operating through an effect on the original explant and not directly in culture. Sears and Deckard (1982) reported that the regenerative activity of calli were affected by the age of the embryos from which they were derived. These variations were reported for calli that originated from embryos which differed in age by 2–3 days. Around the two week stage in grain development the embryo is growing and differentiating rapidly, these time intervals represent major differences in developmental stages which can be recognised morphologically. All of the embryos we cultured were of similar size and morphology to reduce any variation that might result from differences in the degree of explant development. It is impossible to totally control for this factor as we cannot be sure that the cytoplasm does not affect embryo development/physiology – and hence callus response – in a way that cannot be detected visually. In the final analysis if such an effect existed it would still be an effect of the cytoplasm on the tissue culture response, albeit indirect. The examination of cytoplasmic effects in other culture systems using different explants might help resolve the issue of direct/indirect effect.

On the basis of our results it seems unlikely that cytoplasmic substitution will provide a simple means to improve regeneration efficiencies from recalcitrant wheat genotypes. However a detailed examination of the specific effects of the interactions between a range of cytoplasms and nuclear genomes on tissue culture response might provide a means of improving tissue culturability and especially regeneration, through the exploitation of specific nuclear/cytoplasmic interactions. In the longer term regeneration of cytoplasmic lines may be useful in deriving novel cytoplasmic variation, a process which is extremely difficult to accomplish by existing mutagenic techniques. Recent evidence (Matzinger and Burk 1984) suggests that tissue culture will provide a means of separating linked cytoplasmic genes and performing cytoplasmic mutagenesis. Our demonstration of the significant effect that the cytoplasm can have in tissue culture should lead to the investigation and exploitation of such cytoplasmic effects in other tissue culture systems.

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