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In vitro response of leaf tissues from *Lolium multiflorum* – a comparison with leaf segment position, leaf age and in vivo mitotic activity

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Summary. Immature gramineous leaves provide a convenient system for comparing the response of cells in culture with their state of differentiation. Callusing frequency is compared with leaf segment position, leaf age and in vivo mitotic activity in *Lolium multiflorum*. (1) In a succession of one millimeter sections from the immature leaf base, callus was formed from the first and second sections but not the third or subsequent sections. The frequency of those explants callusing decreased with distance from the base of the leaf and with leaf age (or leaf extension growth). (2) In vivo, the proportion of cells in mitosis declined from around 10–14% at the base of young leaves to zero at 5 mm from the base and beyond. Mitotic activity also declined in leaves as they aged, and dividing cells were not observed in leaves 30 days from initiation or older. (3) A high frequency of callus formation was associated with a high mitotic index in the explant. But for corresponding mitotic indices, cells further away from the leaf base were less responsive in culture. (4) It is proposed that cells are becoming differentiated even in highly meristematically active regions of the leaf and concomitantly losing their ability to respond in culture.

Key words: Callus – Leaf culture – Mitotic index – *Lolium multiflorum* – Italian ryegrass

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Introduction

One of the main factors limiting the genetic transformation of a range of plant species, especially members of the Gramineae, is the difficulty of recovering whole plants from single cells.

The genetic transformation of protoplasts isolated from suspension cultures of *Lolium multiflorum* (Potrykus et al. 1985) *Triticum monococcum* (Lorz et al. 1985) and maize (Fromm et al. 1986) has been reported recently and there are now many examples of the recovery of plants from genetically transformed dicotyledonous cultures (e.g. Paszkowski et al. 1984; Horsch et al. 1985). While gramineous suspension cultures are useful for testing the expression of transforming DNA and for determining the parameters important for transformation, they are generally difficult to maintain in a morphogenic state. It is possible to regenerate plants from some gramineous suspension cultures but morphogenic potential is often lost within a few weeks from culture initiation.

There is no clear indication of why plants can be recovered from single cells and disorganised cell cultures in many solanaceous and other dicotyledonous species, whereas in the Gramineae this is, at best, difficult. One important difference between gramineous and dicotyledonous species is that the former do not have a comparable wound reaction. Once the gramineous cell has stopped dividing and is committed to a particular function in the plant, there is no stimulation of cell division following wounding (Dale 1983). The capacity for cell division in the Gramineae is maintained in the meristematic regions to give new leaves and tillers but not in differentiated regions for the repair of damaged tissues.

The developing gramineous leaf provides convenient material to study the relationship between cell differentiation and in vitro response. Cells become progressively more differentiated from the base of the young leaf to the tip. At the base there is the intercalary meristem responsible for providing new cells for leaf extension growth. Moving up the leaf, the cells lose their meristematic activity, they differentiate and eventually take up their various functions within the mature leaf.

The progression of in vitro response in the immature gramineous leaf has been examined in various species (Dale

1983; Wernicke and Milkovits 1984) and Wernicke and Brettell (1982) have related this response to the level of mitotic activity.

The aim of this work was to use *Lolium multiflorum* to investigate the relationships between (a) the position of cells in, and the age of, developing leaves, (b) callusing response of leaf segments in vitro and (c) mitotic activity and mitotic stage in corresponding leaf segments in vivo.

Materials and methods

A population of *Lolium multiflorum* L. selected over several generations for high tissue culture response was used throughout (Dalton and Dale unpublished). All plantlets were grown from seeds surface sterilized for 15 min in 100% sodium hypochlorite (10–14% w/v available chlorine) with 1 drop of Tween 80/100 ml and washed six times in sterile water. Seeds were allowed to stand in sterile water overnight (16 h) before being transferred aseptically to culture medium. The plants were grown in glass jars (28 mm diameter × 85 mm high) with polypropylene screw caps on MS (Murashige and Skoog 1962) basal medium with 3% sucrose and solidified with 5 g l^{-1} Sigma agar. The medium was adjusted to pH 6 and autoclaved at 121 °C for 15 min. The plants in their culture vessels were incubated in a controlled environment room at 20 °C and illuminated with cool white fluorescent tubes at an intensity of 90 μE m^{-2} s^{-1} giving a 12 h light/12 h dark photoperiod.

Culture response and cell division were studied in plants 3, 5, 7, 9, 12, 15, 20, 25 and 30 days old. First (oldest), second, third and fourth leaves were examined as they appeared. Forty plants were selected at each plant age for determining both culture response and mitotic activity.

The shoots consisting of 1–4 rolled leaves were cut transversely into 1 mm long sections in a clean sterile Petri dish with a sheet of millimeter squared paper underneath to allow sizing during dissection. The rolled leaves were separated under a stereomicroscope and those for culturing were placed on MS basal medium with 3% sucrose, 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 0.2 mg l^{-1} 6-benzylaminopurine, 0.1 g l^{-1} casein hydrolysate in an order corresponding to their position in the shoot. The leaf pieces were cultured in 9 cm Petri dishes (agar, pH and autoclaving as above) in continuous light at 0.3 μE m^{-2} s^{-1} and a temperature of 25 °C. The culture response was determined by carefully checking through a stereomicroscope after 4 weeks of culture and is presented as the percentage of the 40 sections with callus formation.

Mitotic activity was determined from the leaf base in one millimeter sections up to 6 mm; cell division was not observed in sections beyond this. The leaf pieces were fixed (after sectioning) for 24 h in 3 : 1 ethanol : acetic acid or until the segments were no longer green. They were then washed in distilled water and fixed for 1 h in 4% formaldehyde in neutral phosphate buffer. This double fixation was used to give satisfactory staining. After fixation sections were washed in distilled water and hydrolysed for 12 min in 1 N hydrochloric acid at 60 °C and stained for 2 h in Feulgen stain (pH 2.2). The sections were macerated in a drop of glycerol on clean microscope slides and squashed under a coverslip without heating. Forty segments were examined per segment position and plant age, and at least 1,200 nuclei per section were observed. The proportions of cells in mitosis and the proportions of cells in early prophase to late telophase were determined and expressed as follows:

Mitotic index = (no. of dividing cells/total no. of cells observed) × 100

Prophase index = (no. of cells in prophase/total no. of cells observed) × 100

and similarly for metaphase index and ana-telophase index (anaphase and telophase cells combined).

Results

Culture response

The callusing response for the 1 millimeter leaf segments from leaf numbers 1–4 and plants 3–30 days old is presented in Table 1. Callusing response declined dramatically from the first to the second millimeter segment and no callus was formed in the third millimeter segment. There was also a sharp decline in callusing response with increasing length and age of the leaf. A correlation analysis between leaf length and culture response for the first millimeter segment of leaf number 1 gave $r = -0.92$ ($P < 1\%$) and leaf number 2 is $r = -0.98$ ($P < 0.1\%$) were both statistically significant.

Mitotic activity

In order to compare the level of mitotic activity in the leaf with segment position, leaf length and culture re-

Table 1. The percentage of leaf segments forming callus (culture response) in culture after excision from leaf numbers 1–4 of plants 3–30 days after their sowing

Leaf no.	Plant age (days)	Mean leaf length (mm)	% Leaf segments callusing 0–1mm[a]	1–2 mm
1	3	11.3	75.0	32.5
	5	40.7	77.6	25.0
	7	66.2	67.5	10.0
	9	89.3	62.5	2.5
	12	110.2	40.0	0
	15	129.9	12.5	0
	20	147.2	4.0	0
	25	162.8	0	0
	30	164.3	0	0
2	9	9.8	77.5	17.5
	12	19.2	75.0	12.5
	15	41.2	62.5	5.0
	20	64.2	42.5	0
	25	98.7	12.5	0
	30	139.3	7.5	0
3	20	9.2	70.0	20.0
	25	16.8	55.0	15.0
	30	39.2	20.0	0
4	30	10.7	62.5	15.0

[a] Distance of segment from the leaf base; no callus was formed in sections further than 2 mm from the leaf base

sponse, the proportion of cells in mitosis (mitotic index) was estimated in the corresponding leaf segments (Table 2). The mitotic index declined sharply from the base of the leaf to the fourth millimeter segment. Mitotic cells were not observed in the fifth millimeter segment or beyond.

The mitotic index at a given segment also declined as the leaves extended. Meristematic activity was high while the leaves were young and extending rapidly; activity declined and ceased as the leaves became fully extended. A correlation analysis between leaf length and mitotic index of the first millimeter segment gave r=−0.84 ($P<1\%$) for the first leaf and r=−0.98 ($P<0.1\%$) for the second leaf; both were statistically significant.

Table 2. The mitotic index of leaf segments from leaf numbers 1–4 of plants 3–30 days of age

Leaf no.	Plant age (days)	Mitotic index of leaf segments			
		0–1 mm[a]	1–2 mm	2–4 mm	3–4 mm
1	3	9.36	11.15	6.22	1.59
	5	10.39	12.65	4.98	1.04
	7	11.64	10.93	2.69	0.15
	9	10.55	6.32	1.16	0.04
	12	6.73	2.15	1.12	0.84
	15	4.93	1.16	1.07	0.16
	20	2.20	0.14	0	0
	25	2.17	0.23	0	0
	30	0	0	0	0
2	9	11.17	14.76	7.93	1.67
	12	10.23	11.17	4.05	0.22
	15	9.93	10.67	2.19	0.44
	20	4.13	1.06	1.07	0
	25	2.04	0.04	0	0
	30	1.15	0	0	0
3	20	10.32	11.14	4.39	1.05
	25	8.22	9.83	3.16	0.84
	30	3.93	2.16	0.76	
4	30	9.99	8.76	3.33	1.45

[a] Distance of segment from the leaf base; dividing cells were not observed in segments further than 4 mm from the leaf base

Relationship between culture response and mitotic index

A linear regression analysis of culture response onto mitotic index (both converted to angles) using the data from all four leaves, different plant ages and leaf segment positions is positive (b=3.96; $P<0.1\%$) and highly significant. The correlation coefficient was r=0.93 ($P<0.1\%$). In general, therefore, the higher the mitotic index the greater the response in culture. However, a closer examination of the data indicates that for a given mitotic index there is a difference in response between the first and second millimeter segments. The second segment is less responsive in culture (Fig. 1). A joint regression analysis of variance (Table 3) shows that:

(a) The joint regression component is highly significant confirming that overall there is a decline in culture response with declining mitotic index;
(b) The heterogeneity of means is significant showing that overall, the second millimeter was less responsive than the first millimeter segment and;
(c) The heterogeneity of regressions is significant showing that there is a difference in the relationship of culture response and mitotic index between the first and second leaf segments.

Culture response is therefore not simply related to the proportion of cells dividing.

Relationship between culture response and the stage of mitosis

There is active cell division at the base of the growing leaf which declines and stops in higher leaf segments as cells differentiate. At the leaf base a high proportion of the *dividing cells* are in prophase and a low proportion

Table 3. Joint regression analysis of variance (after converting to angles) of culture response onto mitotic index, prophase index, metaphase index and ana-telophase index

	Mitotic index		Prophase index		Metaphase index		Ana-Telophase index	
	VR[a]	P*	VR	P	VR	P	VR	P
Joint regression	143.4	<0.1	115.1	<0.1	41.3	<0.1	85.6	<0.1
Heterogeneity of regression	16.2	<0.1	10.3	<1	4.0	5.3	17.0	<0.1
Heterogeneity of means	94.5	<0.1	–[b]	–	–	–	–	–

[a] Variance ratio
[b] Mean culture response values are the same as for mitotic index
* % probability

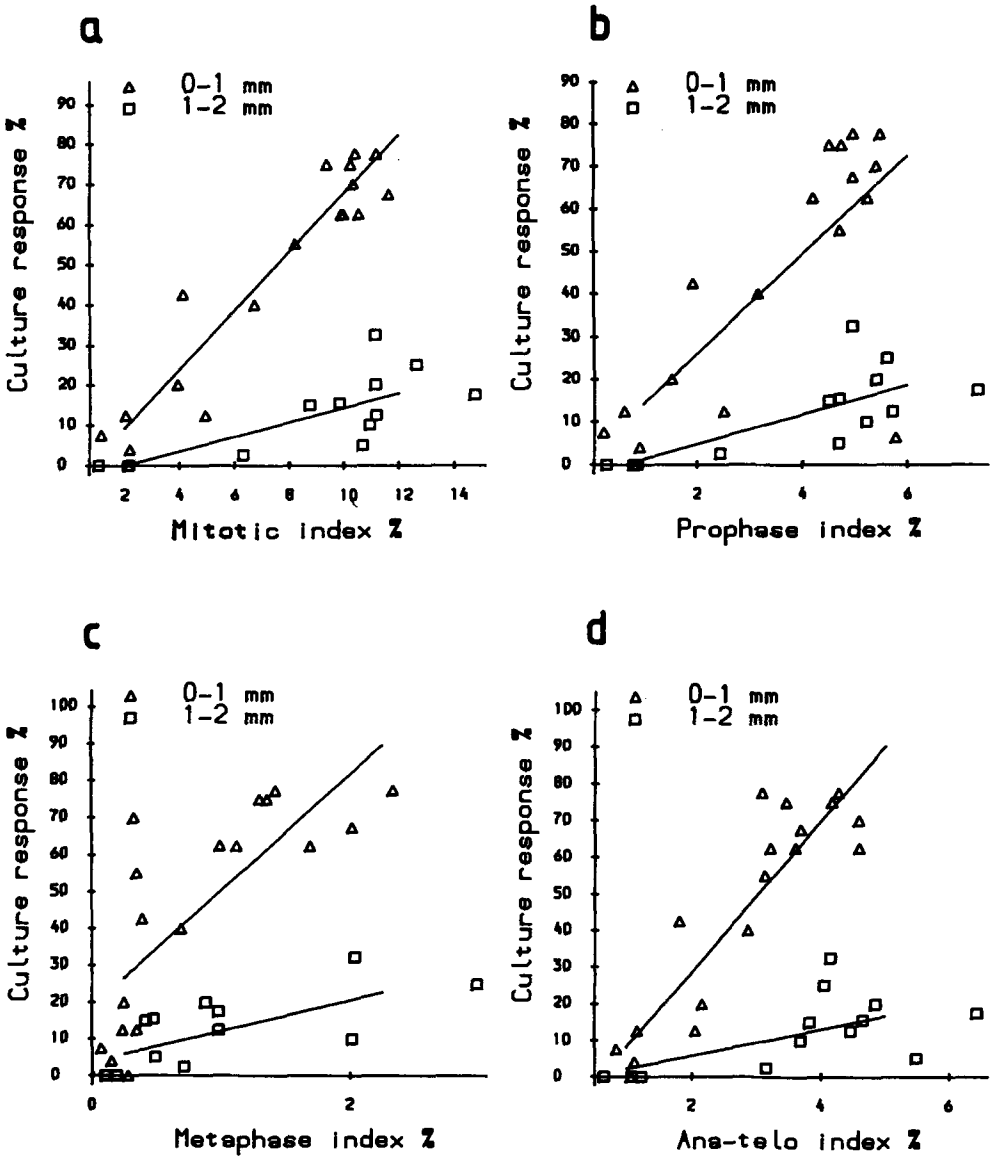

Fig. 1. Culture response of the four leaves and different plant ages plotted against (**a**) mitotic index (**b**) prophase index (**c**) metaphase index and (*d*) ana-teleophase index for the first (△) and second (□) millimeter leaf segments. Separate regression lines are shown

at ana-telophase. Moving along the leaf from the base, the proportion of dividing cells at prophase declines and the proportion at ana-telophase increases and eventually declines with the cessation of cell division. For example, in the first leaf of a 5-day old plant the percentages of dividing cells at prophase are 48, 44, 21 and 18 for the first, second, third and fourth millimeter sections, respectively. Whereas the corresponding percentages for ana-telophase are 30, 32, 75 and 66.

To determine whether cells at a particular phase of cell division are more responsive than others, the number of cells at prophase, metaphase and ana-telophase was estimated (Table 4). Culture response is plotted against the proportion of the total number of cells in the three stages in Fig. 1 b, c and d. A joint regression analysis of variance (Table 3) shows that: 1) there is an overall decline in culture response with declining prophase, metaphase and ana-telophase indices and 2) there is a difference in the relationship of callusing response and the two indices between the first and second leaf segments. The heterogeneity of regressions between callusing response and metaphase index was of borderline significance.

It is evident therefore, that callusing response is not directly related to the proportion of the total number of cells at any stage of mitosis.

Table 4. The prophase, metaphase and anaphase-telophase indices of leaf segments from leaf numbers 1–4 of plants 3–30 days of age

Leaf no.	Plant age (days)	0–1 mm[a]			1–2 mm			2–4 mm			3–4 mm		
		Pro	Met	A-T	Pro	Met	A-T	Pro	Met	A-T	Pro	Met	A-T
1	3	4.52	1.36	3.47	4.96	2.04	4.15	1.13	0.97	4.12	0.11	0.14	1.34
	5	4.97	2.33	3.09	5.62	2.98	4.05	1.05	0.22	3.71	0.19	0.16	0.69
	7	4.96	2.02	3.69	5.22	2.02	3.69	0.92	0.14	1.63	0.02	0.03	0.10
	9	5.25	1.69	3.61	2.45	0.71	3.16	0.24	0.14	0.78	0.01	0.01	0.03
	12	3.17	0.69	2.87	0.89	0.19	1.08	0.34	0.07	0.71	0.14	0.09	0.61
	15	2.52	0.35	2.06	0.50	0.08	0.58	0.26	0.09	0.72	0.02	0.02	0.12
	20	0.93	0.15	1.12	0.02	0.03	0.09	0	0	0			
	25	0.82	0.28	1.07	0.03	0.02	0.18						
	30	0	0	0	0	0	0						
2	9	5.48	1.42	4.27	7.36	0.97	6.43	2.35	0.55	5.03	0.22	0.23	1.22
	12	4.76	1.30	4.17	5.72	0.98	4.47	0.87	0.34	2.86	0.04	0.03	0.15
	15	4.22	1.12	4.59	4.69	0.49	5.49	0.56	0.21	1.42	0.09	0.07	0.28
	20	1.94	0.39	1.80	0.31	0.10	0.65	0.13	0.09	0.85	0	0	0
	25	0.63	0.24	1.17	0.01	0	0.03	0	0	0			
	30	0.24	0.07	0.84	0		0						
3	20	5.40	0.33	4.59	5.41	0.88	4.85	2.01	0.28	2.10	0.19	0.07	0.79
	25	4.73	0.35	3.14	4.71	0.48	4.66	1.40	0.23	1.53	0.13	0.08	0.63
	30	1.52	0.25	2.16	0.82	0.11	1.23	0.15	0.08	0.53	0	0	
4	30	5.78	0.99	3.22	4.51	0.42	3.83	0.92	0.21	2.20	0.15	0.10	1.20

[a] Distance of segment from the leaf base

Discussion

The recovery of plants from cultured cells in the Gramineae involved having an understanding of, and being able to manipulate two processes: first the stimulation of cell division to form callus and second the regeneration of plants from that callus. The ability of gramineous cells to undergo these two processes depends on several factors e.g. plant genotype, plant growth conditions, culture media, culture conditions and of particular importance, the state of cell differentiation. Mature leaf tissues containing fully differentiated cells cannot be induced to form callus and, mature mesophyll protoplasts isolated from gramineous leaves have not given repeatable, sustained cell division and callus formation (Potrykus et al. 1976; Dale 1983). In contrast, the cells within explants from the intercalary meristem of immature gramineous leaves are capable of callus formation and in many species, morphogenesis (Dale 1983; Wernicke and Milkovits 1984).

From the results presented, the ability of the leaf tissue to form callus in culture depends on both the position of the leaf segment (explant) and the age (and overall length) of the leaf. The frequency of explants forming callus is highest at the base of the immature leaf and declines sharply to the third millimeter and beyond where no callus is formed. Callusing frequency from leaf base sections also declines with increasing age of the leaf.

This decrease in callusing response is associated with two phenomena in the developing leaf: cell differentiation and leaf extension. Both these processes involve the reduction and eventual cessation of cell division when (a) cells from the intercalary meristem differentiate and take up their various functions in the mature leaf and (b) at the end of leaf extension when cells of the intercalary meristem cease meristematic activity.

Because of the association between culture response and meristematic activity, it is important to compare culture response directly with the mitotic index of the corresponding leaf segment in vivo. In *Sorghum bicolor*, Wernicke and Brettell (1982) observed that high callus fresh weight from immature leaf sections was associated with high mitotic indices. A review of the literature on gramineous tissue culture also shows that callus induction is generally from meristematically active explants (Dale 1983). Is the frequency of callus formation simply related to the proportion of dividing cells in the explant? Do all dividing leaf cells have the same probability of forming callus in culture?

The results in *Lolium* show that for a given leaf segment the higher the mitotic index, the higher the frequency of callus formation. However, callusing frequency does not directly and simply relate to the level of mitotic activity; for comparable mitotic indices the second millimeter leaf segment is less responsive than the first. A similar comparison of the number of cells at prophase, metaphase and ana-telophase with callusing frequency shows that the second millimeter is also less responsive than the first, indicating that it is not a specific stage within mitosis that is able to respond in culture.

The evidence suggests, therefore, that mitotically active cells within the upper regions of the intercalary meristem of the leaf are already passing into the early

stages of differentiation, becoming committed to their functions within the leaf and inaccessable to the influence of the in vitro conditions. A knowledge of the nature of this differentiation process is important to improving our understanding of how to manipulate gramineous cells in culture. The relationship between the stage of differentiation, DNA content, phase within the DNA replication cycle in vivo and morphogenic response in vitro will be considered in later publications.

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