

Developmental genetics of leaf formation in *Lolium*

2. ANALYSIS OF SELECTION LINES

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1. INTRODUCTION

In the first paper of this series, I have described the relationships between the timing of the phases of development of successive leaves on a shoot of ryegrass (Edwards, 1967). To recapitulate briefly, the main points arising from this study were two. First, during the early development of an individual leaf there was a fairly sudden transition in its growth rate which occurred when it was about 1 mm. long. This transition I have called the 'unfolding' of the leaf; before this the primordium grows slowly, afterwards the leaf grows rapidly and elongates at a rate which remains more or less linear until just before it matures. The second point is that the time of unfolding of a leaf was almost perfectly synchronized with the maturation of the next older leaf on the same side of the apex. Thus at any given time only two leaves (one on each side of the apex) were actively elongating on any shoot, while the duration of elongation of an individual leaf was negatively correlated with the rate of leaf unfolding, i.e. the rate at which successive leaves unfold at the apex.

This association was consistent over two species of *Lolium*, *L. multiflorum* and *L. perenne* (although each species was represented by only one variety), and over two environments, January and July sowings in the glasshouse. It led to the prediction that on the one hand a response to selection for larger leaves, in so far as it was due to an increase in the duration of leaf elongation, would be likely to be accompanied by a reduction in the rate of leaf appearance, while on the other hand, selection for rapid rate of leaf appearance would be likely to produce smaller leaves by reducing the duration of leaf elongation.

Edwards & Cooper (1963) reported the results of selection for leaf size or rate of leaf appearance in one population of *L. multiflorum* and two populations of *L. perenne*. In all cases except one there were negative correlated responses between leaf size and rate of leaf appearance. The present paper reports the detailed developmental analysis of these selection lines to see how well the model of leaf formation on a shoot outlined above fits responses to selection for leaf characters.

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2. MATERIALS AND METHODS

The selection lines used were those reported on by Edwards & Cooper (1963), selection having been continued for a further two generations up to the 5th. For these two further generations the mating system used was double first-cousin as before, giving two families in each line at each generation. Both families in each line at the 5th generation were grown in this experiment, but in most cases there were no significant differences between families within lines and so the means of the lines are quoted. Two lines within one of the *Lolium perenne* populations (Irish), the small leaf size line and the slow rate of leaf appearance line, had died out by the 5th generation and so were not available for developmental analysis.

Throughout the rest of this paper the three populations which were the bases for selection will be referred to as Italian (*L. multiflorum*), Irish, and Hunsballe (*L. perenne*). The lines within each population will be referred to as:

Large: Line selected for large leaf size

Small: Line selected for small leaf size

Fast: Line selected for fast rate of leaf appearance

Slow: Line selected for slow rate of leaf appearance.

The plants were grown in John Innes compost in boxes in a glass-house in the January of 1965. There were three blocks, each containing eight plants of each family (i.e. sixteen plants of each line). Five of the eight plants were used for detailed growth records, the remaining three being harvested for dissection of the apex of the main shoot at 29, 49 and 87 days after sowing.

Detailed records were made on the growth and final dimensions of leaves 3, 4, 5, and 6 on the main shoot. Dates of appearance and of maturation, the rate of elongation at appearance and the final length and width were recorded directly. The date of unfolding and the duration of rapid elongation of each leaf were estimated as in Edwards (1967).

The rates of unfolding, appearance and maturation of successive leaves were also computed from the appropriate dates. Mature 6th leaves were harvested and dimensions of cells of the lower epidermis were measured after preparation of the leaf according to the method of Clarke (1960).

The data obtained from this experiment have been compared with the results of the 3rd generation lines reported by Edwards & Cooper (1963), the latter experiment however was recorded after an August sowing.

3. RESULTS

(i) Comparison with 3rd generation

The leaf dimensions and rates of leaf appearance of the 5th generation lines are compared in Table 1 with the 3rd generation data reported by Edwards & Cooper (1963). The 5th generations of the Irish small and slow lines were not available, but

the 3rd generation data for Irish show that the difference between the large and small lines was the inverse of that between the fast and slow lines. The two comparisons of large versus small and slow versus fast show that in each case the former had longer and wider leaves and a slower rate of leaf appearance than the latter. Thus it seems reasonable to treat the large and fast lines as complementary, the one having large leaves and slow rate of leaf appearance, the other having small leaves and fast rate of appearance.

Table 1. *Leaf dimensions and rates of appearance of new leaves in lines selected for leaf size or rate of leaf appearance*

Population	Generation	Line	Leaf size (mm. ²)	Leaf length (mm.)	Leaf width (mm.)	Rate of leaf appearance (lvs/week)
1. Selection for Leaf Size						
<i>L. multiflorum</i> (Italian)	3	Large	1841	276	6.6	0.44
		Small	951	204	4.6	0.70
	5	Large	1494	226	6.5	0.81
		Small	588	114	5.1	0.97
<i>L. perenne</i> (Irish)	3	Large	1206	253	4.7	0.82
		Small	517	149	3.4	0.98
	5	Large	838	174	4.7	0.93
		Small*				
<i>L. perenne</i> (Hunsballe)	3	Large	1114	223	5.0	0.71
		Small	746	202	3.6	0.88
	5	Large	689	140	4.8	0.88
		Small	321	107	3.0	1.03
2. Selection for Rate of Leaf Appearance						
<i>L. perenne</i> (Irish)	3	Fast	362	127	3.0	1.30
		Slow	724	170	4.1	0.54
	5	Fast	201	75	2.7	1.26
		Slow*				
<i>L. perenne</i> (Hunsballe)	3	Fast	947	229	4.0	0.97
		Slow	970	215	4.4	0.64
	5	Fast	625	156	4.0	1.03
		Slow	663	150	4.2	0.81

* Not available.

There are overall differences in leaf size and rate of leaf appearance between the two sets of data. The average rate of leaf appearance for the 3rd generation experiment (excluding the Irish small and slow lines) is 0.81 leaves per week, while for the 5th generation it is 0.97. For leaf size, the 3rd generation has larger leaves (1017 mm.² compared with 677). This leaf size difference is almost entirely due to the 3rd generation material having longer leaves, there being little difference in width. Since the data for the two generations were not collected simultaneously but in two

separate experiments, the differences between them may well be environmental. In fact this is highly probable because the 3rd generation was sown in August while the 5th generation was sown in January. Some measure of the environmental effects can be made from the data provided by unselected samples of the three base populations which were grown with each of these two populations. The averages over the three for each experiment were as follows:

	Leaf size	Rate of leaf appearance
3rd generation experiment	1243 mm. ²	0.86 lvs/week
5th generation experiment	652 mm. ²	1.01 lvs/week

These changes closely parallel those between the means of all the selection lines in the two experiments.

Allowing for this absolute difference between the results for the two generations, the difference between the two extreme lines selected for any character is very similar at the 3rd and 5th generations. For example, the difference in leaf size between the large and small lines in Italian is 890 mm.² at generation 3, and 906 mm.² at generation 5. The comparable figures for the Hunsballe large and small lines are 368 mm.² and 368 mm.². The same relationship between the 3rd and 5th generations hold for most other characters, although the difference between the fast and slow lines in Hunsballe was actually less at the 5th generation than at the 3rd (0.22 leaves per week compared to 0.33). Whether such changes have any significance is doubtful because the generations are confounded with seasonal differences. But it certainly appears that there has been little response to selection between the 3rd and 5th generations, although since the mean leaf size in the 5th generation experiment was only half that in the 3rd generation experiment, an absolute differential between the extreme lines of the same magnitude represents a relative differential which is considerably greater at the 5th generation.

All the lines show that the correlated responses at both generations are similar. In all three populations which were selected for leaf size a change in size has been a result of parallel changes in both length and width. But in Hunsballe selection for rate of leaf appearance has caused no change in leaf size or in length or width at either generation.

(ii) *Rates of formation of leaves*

The total number of primordia initiated by each of three dates are shown in Table 2. The only instances of statistically significant differences occurring between lines within populations at specific dates are at 29 days after sowing in Italian and at 87 days in Irish. The former is significant at only a low level of probability ($0.05 > P > 0.01$), while the latter may have been affected by the fact that at this third date some plants in Irish fast lines were initiating reproductive structures at the apex. Where this was obvious at dissection the plant was discarded but the results recorded may be unreliable because the onset of reproductive development is associated with a sudden increase in the rate of initiation of primordia.

Table 2. Rates of initiation of primordia. Total number of primordia and expanding and mature leaves at three dates
Generation 5

Selection line		Days after sowing			Mean
		29	49	87	
Italian	Large	6.25	12.50	20.4	13.05
	Small	7.25	13.50	21.7	14.15
Irish	Large	6.25	12.25	18.8	12.43
	Fast	7.00	13.25	23.3	14.52
Hunsballe	Large	6.00	11.25	16.2	11.15
	Small	6.50	11.50	16.0	11.50
	Slow	6.50	10.75	16.8	11.35
	Fast	7.00	12.00	18.2	12.33
Standard error		0.25	0.59	0.73	0.32

Although individual differences between lines at particular dates may be small they do appear to be consistent over the three harvests. In fact, in all comparisons of pairs of lines the line with the faster rate of leaf appearance has the greater number of primordia. When the line means, calculated over all three dates, are compared this difference has a less than 0.01 probability of being due to chance in each of the four pairs except for the comparison of Hunsballe large and small lines. Thus it does appear that selection has produced some changes in the rates of primordia initiation, although these differences are generally small. In fact, although these differences between lines for the total number of primordia initiated at particular dates do seem to be real, Table 2 shows that the differences between lines at 87 days were of the same order of absolute magnitude as those at 29 days. This suggests that differences which do exist were established very early and that during the interval between these dates the rate of initiation did not differ appreciably.

Although primordia may be initiated at the apex at more or less the same rate in different lines, it does not follow that they will develop into leaves at the same rate. The estimated dates of unfolding (i.e. transition from slow primordial growth to rapid leaf elongation) for leaves 3 and 6 on the main shoot are shown in Table 3. In each case except leaf 3 in Italian there is significant variation between lines within populations, and in all comparisons the line with the faster rate of leaf appearance has the earlier date of unfolding. The differences between lines are more marked for leaf 6 than for leaf 3, showing that the rates of unfolding also differ between lines.

The rates of unfolding, together with the rates of appearance and of maturation, are also presented in Table 3. The mean rates over all lines are 1.00, 0.97 and 1.01 leaves per week respectively for unfolding, appearance and maturation. They are obviously very similar although the rate of appearance is significantly lower ($P < 0.01$) than the other two. The ranking of the various lines is also very similar for the three rates, and in general the values themselves are very similar with the

exception of the two pairs of leaf size lines. In Italian the difference between the large and small lines is 0.09 leaves per week for rate of unfolding, 0.16 for rate of appearance and 0.30 for rate of maturation. In Hunsballe the corresponding figures are 0.08, 0.15 and 0.26. In both cases this represents a statistically significant interaction between lines and the measure of rate of formation of leaves ($P < 0.001$). Despite these differences in rates for the leaf size lines, it is clear that the rates of leaf appearance and maturation are largely determined by the rates of leaf unfolding.

Table 3. *Days from sowing to unfolding of leaves 3 and 6. Rates of unfolding, appearance and maturation of leaves on main shoot (leaves per week)*

Generation 5

Selection line		Days to unfolding		Rate of unfolding	Rate of appearance	Rate of maturation
		Leaf 3	Leaf 6			
Italian	Large	26.3	49.9	0.89	0.81	0.79
	Small	26.3	47.9	0.98	0.97	1.09
Irish	Large	27.0	49.1	0.95	0.93	0.99
	Fast	24.4	42.7	1.22	1.26	1.31
Hunsballe	Large	28.9	51.3	0.93	0.88	0.87
	Small	26.4	47.2	1.01	1.03	1.13
	Slow	28.3	53.8	0.83	0.81	0.82
	Fast	26.1	46.1	1.16	1.03	1.09
Standard errors		0.53			0.02	

(iii) *Duration and rates of growth of individual leaves*

Table 4 shows that in all comparisons between extreme lines, larger leaves are associated with a longer duration of elongation while the duration is reduced in lines with a faster rate of leaf appearance. This reduction in fast lines agrees with that predicted from the relationship between the duration of elongation of an individual leaf and the interval between the unfolding of successive leaves.

Table 4. *Duration and rates of elongation of leaf 6 and cell details*

Generation 5

Selection line		Leaf 6 length mm.	Duration of elongation (days)	Rate of elongation (mm./day)	Cell length (μ .)	Estimated number of cells in length
Italian	Large	226	20.1	15.4	633	357
	Small	114	13.7	11.7	695	164
Irish	Large	174	14.6	14.7	584	298
	Fast	75	11.5	9.7	430	174
Hunsballe	Large	140	16.3	13.5	611	229
	Small	107	13.2	11.2	527	203
	Slow	150	16.3	14.1	503	298
	Fast	156	13.9	16.0	620	252
Standard error		5.0	0.49	0.53	30.0	20.4

The duration of elongation is a component of final leaf length and a reduction will produce a shorter leaf unless compensated by an increase in the rate of elongation. Table 4 shows that in all comparisons between large and small lines the former have both longer durations and higher rates of leaf elongation. But a comparison between the fast and slow lines in Hunsballe shows that the expected reduction in duration in the fast line was compensated for by an increased rate. From Table 1 it is clear that this compensation is complete since the lines do not differ in final length.

Turning to the epidermal cell data in Table 4, there is no consistent pattern over populations. The differences in leaf length between the large and small lines appear to have different cellular bases in the three populations. In Italian the difference is due to cell number only; in Irish to both cell number and cell size; and in Hunsballe to cell size only (although in this case the significance of the cell size difference is only marginal with a probability of 0.05). The Hunsballe fast and slow lines are of interest because the compensating processes of duration and rate of leaf elongation, which give the same final leaf length in the two lines, appear to be reflected by the cell length and cell number data. The fast line has the shorter duration but the higher rate of leaf elongation, and it also has fewer but longer cells than the slow line. This suggests that the duration of elongation is dependent on the length of time cell division continues and that the rate of elongation of the leaf is dependent on the rate of enlargement of cells. But the data for the other lines do not support the idea that these are universal relationships and it is much more likely that particular rates and durations of leaf elongation could be produced by any one of a number of combinations of rates and durations of cell division and cell enlargement.

(iv) *Sequence of leaf formation in successive leaves*

The data presented so far show that lines with a greater duration of leaf elongation have a slower rate of leaf unfolding. This suggests that the basic association between the time of maturation of leaf (n) on a shoot and the time of unfolding of leaf ($n + 2$) has not been greatly disturbed by selection. The results from this experiment provide two such comparisons: (i) between maturation of leaf 3 and unfolding of leaf 5, and (ii) between the maturation of leaf 4 and unfolding of leaf 6. The degree of coincidence for each of these two associations can be seen in Fig. 1. In all the Italian and Irish lines the coincidence is high as it is also in the Hunsballe small and fast lines. In the Hunsballe large and slow lines, however, there are positive intervals between the maturation and unfolding of the appropriate leaves of 1.7 and 1.1 days, and 2.2 and 1.5 days respectively. There were no overall differences between the intervals measured at the two growth stages, but the variation between lines was marginally statistically significant ($P \approx 0.05$). Clearly such variation between lines as did occur was due to the Hunsballe large and slow lines (with a mean interval of 1.625 days) differing from all the other lines (mean interval 0.075 days).

It appears, however, that selection has not greatly disturbed the association between the times of maturation of a leaf and of unfolding of the next younger leaf on the same side of the apex, despite the fact that the lines differ considerably in rate of leaf unfolding and in duration of leaf elongation.

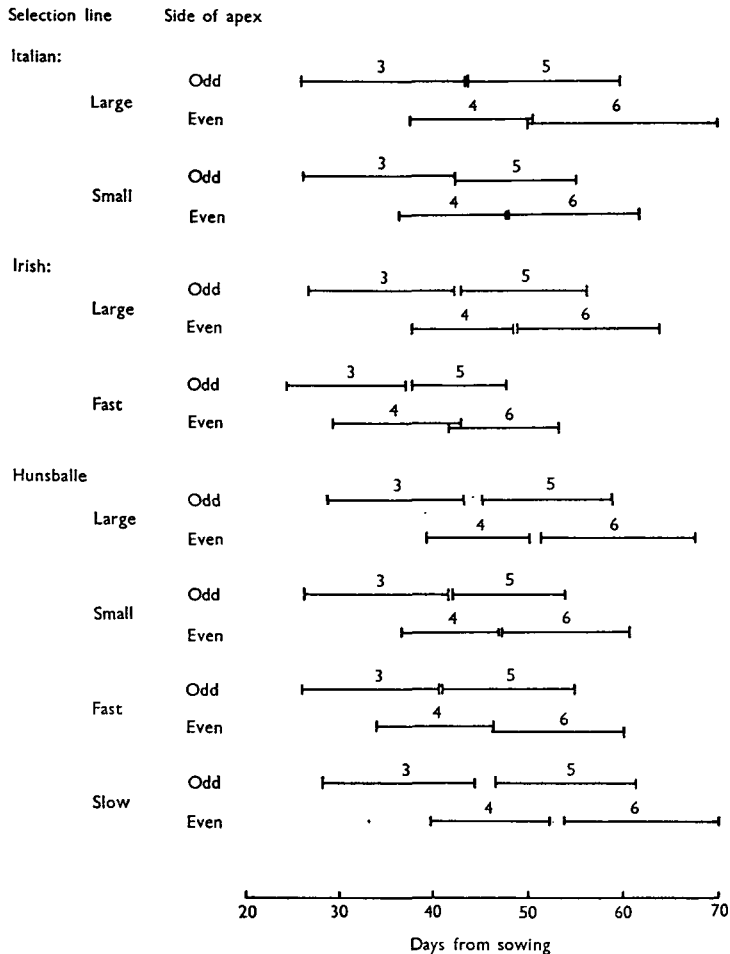


Fig. 1. Relationships between times of unfolding and of maturation of successive leaves on shoot in selection lines. Generation 5.

Standard errors: (i) Dates of unfolding or maturation = 0.51 days.

(ii) Interval between maturation of leaf (n) and unfolding of leaf ($n + 2$) = 0.72 days.

4. DISCUSSION

The close relationship in time between the maturation of a leaf (i.e. the cessation of growth) and the unfolding of the next younger leaf on the same side of the apex (i.e. the transition from slow primordial growth to rapid leaf elongation) appeared to be a fundamental one because it was consistent over two species in two environments. This suggested that it was probably well buffered against genetic change and would be unlikely to be greatly affected by selection for characters expressed at later stages of development. The results reported in the present paper show that this prediction has been very largely fulfilled and that selection for either leaf size or for rate of leaf appearance has had little effect on the relationship between

maturation and unfolding of successive leaves on one side of the apex. The net result of this association is that the number of leaves elongating rapidly at any time has remained constant at two (one on each side of the apex) in all the selection lines.

The developmental sequence which produces this close association and which appears to be so well buffered against genetic change is intriguing but not understood. Various workers have shown that the transition from slow primordial growth to rapid leaf growth in Gramineae is associated with the development of vascular connexions between the developing leaf and the vascular network of the shoot (Sharman, 1945; Esau, 1954; Williams, 1960). But the cause of the association between this event and the cessation of growth of the next older leaf on the same side of the apex is unknown, although experiments which will be reported elsewhere and which used some of these lines in controlled conditions of light intensity, temperature and external nitrogen supply suggest that the association is not merely a result of passive competition for nutrients, but is rather an active process.

But the regularity of this association does not mean that selection for increased rate of leaf appearance must always be accompanied by reduced leaf size or vice versa. For while a change in the rate of unfolding of new leaves (and, therefore, in their rate of appearance) is always associated with a negative change in the duration of leaf elongation, leaf size is not determined solely by the period of elongation. The duration may affect length but not necessarily width; these two dimensions are positively correlated however, and in these selection lines a change in leaf size is always the product of changes in the same direction in both length and width. But even for leaf length the duration of elongation is not the sole determinant, for the rate of elongation also contributes. In this experiment selection for greater leaf size has increased both the duration and the rate of elongation, and the former change has been accompanied by a reduced rate of leaf appearance. Selection for increased rate of leaf appearance has also increased the rate of unfolding and this has produced the expected reduction in the duration of elongation, but in *Hunsballe* a compensating increase in the rate has cancelled out any decrease in leaf length and, since width is also unaffected, leaf size has not been reduced.

Since the negative correlation between leaf size and rate of leaf appearance is by no means complete, it should be possible to develop selection for rate of total leaf area development on the shoot of which these are components. Some attempt to do this has already been made by selecting for the product of individual leaf size and rate of leaf appearance. But progress has been very slow, as might be expected from the developmental interactions we have seen to be present. It remains to be seen whether a more detailed knowledge of these interactions can help to set up a more efficient selection criterion, such as, for example, a combination of a more rapid leaf appearance rate and a more rapid rate of leaf elongation, based on a more thorough knowledge of the inheritance of these processes which is now being investigated.

Wherever desirable changes in a selection programme are accompanied by undesirable ones, as often happens between components of a character (e.g. Johnson & Aksel, 1959), the question arises whether the correlation is due to linkage or to some sort of physiological limitation. If linkage is the cause then it would be

expected that, unless it is very tight, the association would be recombined occasionally. If there is a physiological limitation due to a developmental or biochemical pathway which is common to both characters, then a negative correlation between them would suggest that the limiting process cannot be improved. This could be because there is no segregation in the population for genes affecting the process or because it is buffered against genetic change. In outbreeding populations like ryegrass homozygosity for all genes affecting a process is unlikely, but if this were the case there would be little hope for improvement. If, on the other hand, the process is buffered, the implication is that segregating in the population are some genes which under certain conditions of background genotype or of environment might give rise to phenotypic variation.

The fact that the close association between the maturation and unfolding of successive leaves on each side of the apex appeared in the three populations studied, together with the obvious developmental proximity of the events, suggests that the association is due to a physiological limitation. The improbability of all genes affecting a character being homozygous in outbreeding populations suggests that buffering of the process must at least be considered as a possibility. It might be feasible to disturb such buffering by changing the environment and so exposing genetic variation. Experiments on *Lolium multiflorum* and *L. perenne* reported by the author (Edwards, 1967) showed that a January as opposed to a July sowing in a glass-house produced no disturbance of the association, but it is possible that more unusual combinations of light and temperature treatments may do so. Experiments in controlled environment rooms are now under way to test this suggestion.

If an environmental change can release this variation it might be possible to select for the limiting process under the disturbing environment to the extent that a threshold is overcome so that at least a part of the selection response is expressed in the normal environment (Waddington, 1959). Of course, if a process is buffered against genetic change it is presumably because this buffering has some advantage under natural selection and care would have to be taken to avoid any deleterious side-effects of a breakdown of the canalization. In the present case, for example, it may be (although this is completely speculative) that the association between maturation and unfolding of leaves ensures that the basic positional relationship of one leaf to another is maintained so that each leaf blade is raised above, but only just above, the preceding leaf.

Developmental analysis may be a useful tool in plant breeding in elucidating the relationships between the components of some character which is showing little response to selection rather than merely identifying such components. These components may show a great deal of additive genetic variation and be capable of considerable response to independent selection, but may be negatively correlated, as is the case with leaf size and rate of leaf appearance in the present study.

SUMMARY

Detailed measurements of leaf growth and leaf dimensions in the seedling stage were made on lines which had been selected either for large or small leaf size or for

fast or slow rates of leaf appearance within one population of *Lolium multiflorum* (Italian ryegrass) and two populations of *L. perenne* (Irish perennial and Hunsballe perennial ryegrass).

Selection for either character had no effect on the rate of initiation of primordia at the apex, but did change the rate at which successive primordia became leaves. This rate of unfolding was very highly correlated with the rate of visible appearance of leaves and in all cases showed a parallel response to selection for the latter, as did also the rate of maturation of leaves. All three rates showed a negative correlated response to selection for leaf size.

Selection for increased leaf size in all cases led to a longer duration of the elongation of an individual leaf, but selection for faster rate of leaf appearance always reduced this duration. The rate of elongation of individual leaves increased under selection for larger leaf size but showed irregular changes under selection for faster leaf appearance, going down in Irish but up in Hunsballe.

Data for dimensions of cells from the lower epidermis showed that changes in leaf length under selection were sometimes associated with changes in cell length, sometimes in cell number and sometimes with both.

Selection had in no case disrupted the close association between the maturation and cessation of growth of a leaf on the one hand, and, on the other, the unfolding from the apex and onset of rapid growth of the next younger leaf on the same side of the apex. Thus in all lines only two leaves (one on each side of the apex) were elongating rapidly at any one time, and an increase in the rate of unfolding was associated with a decrease in the duration of elongation and vice versa.

This association was the basis of the observed negative correlated responses between leaf size and rate of leaf appearance. But the fact that the rate of elongation could change independently of the duration opened up the possibility of setting up a selection criterion which would increase the total rate of leaf area formation.

The value of this kind of analysis of a character complex in a plant-breeding programme is suggested to lie in discovering physiologically or developmentally limiting processes rather than merely identifying morphological components.

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