

COMPARATIVE EFFICIENCY OF DEVELOPMENT UNDER COLD STRESS OF THE TROPICAL LEGUMES *LOTONONIS BAINESII* BAKER AND *STYLOSANTHES HUMILIS* H.B.K.

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Abstract

The efficiency of development of *L. bainesii* and *S. humilis* in response to increasing, specific amounts of cold stress was compared. The plants were grown under controlled environment with favourable lighting and day length regime typical of the advancing autumn season in subtropical regions. Two temperature regimes were imposed, a "normal" regime typical of the field during frosting, and a "chilled" regime, some 10°C lower than the normal.

Both legumes suffered a pronounced check to development, but the greater magnitude of effect was on *S. humilis*, especially with chilled roots. The superiority of *L. bainesii* was apparent from visual observation, was demonstrated in the multivariate sense by numerical techniques, and was confirmed by the principal discrete growth responses. The attributes making the major contribution were: the ratio of root to tops dry weight, the levels of tissue water, and the relative nitrogen content of the tops. Their responses confirmed a greater measure of cold tolerance possessed by *L. bainesii* when compared with *S. humilis*. They also indicated a greater capacity in *L. bainesii* to harden under these conditions of gradually increasing cold stress.

The cold tolerance of *L. bainesii* is a valuable propensity for a tropical pasture legume, and warrants the fullest exploitation both in practice and by further scientific evaluation as a comparison with *S. humilis* at the metabolic level.

I. INTRODUCTION

Tolerance of cold and frosting are desirable qualities for tropical pasture species because cold may impose severe limitations upon their productivity in regions subject to the influx of cold dry air masses. Such regions are a feature of the northern half of Australia. The development of *Lotononis bainesii* Baker in response to specific amounts of cold stress has been described relative to its phosphorus nutrition and the age at which stress was imposed (Gates *et al.* 1973). In that study of the interaction of nutrition, cold, and age, it was concluded that the response to chilling was multiple rather than discrete; for the normal pattern of growth response adapted constantly through time to treatment interaction. It is the aim of the present paper to compare the response to specific amounts of cold of two tropical legume species, *L. bainesii* and *Stylosanthes humilis* H.B.K. (Townsville stylo). The nature of their recognized differences in response has not been determined previously. It may be expected to be a complex of interacting factors.

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S. humilis is a summer-growing annual, native to Central and South America, lat. 20°N.–20°S. of the equator. *L. bainesii* is a summer-growing perennial from Africa, lat. 22–27°S., at 1000–1500 m altitude, areas in which plants are exposed to cold dry winters. Both legumes are important pasture species in the northern half of Australia.

The comparisons of these species were made in a nitrogen-deficient soil in which plants depend primarily upon symbiotically fixed nitrogen. The selected age and level of phosphorus nutrition were found favourable in previous studies for the growth of *L. bainesii* (Gates *et al.* 1973) and for *S. humilis* (Gates *et al.* 1966).

II. MATERIALS AND METHODS

(a) Experimental

L. bainesii and *S. humilis*, inoculated with *Rhizobium* strains CB376 and CB756 respectively, were sown into polythene-lined pots containing 2420 g (oven dry) of a light-textured, solodic soil and were covered with a 1.5-cm layer of acid-washed quartz gravel. All pots received: 0.199 g K_2SO_4 , 0.008 g $CuSO_4 \cdot H_2O$, 0.008 g $ZnSO_4 \cdot H_2O$, 0.0006 g $Na_2MoO_4 \cdot 2H_2O$, and 0.289 g $NaH_2PO_4 \cdot 2H_2O$. The pretreatment temperature was 28°C continuous and light 3500 f.c. per 13½-hr day. Plants were thinned to four per pot.

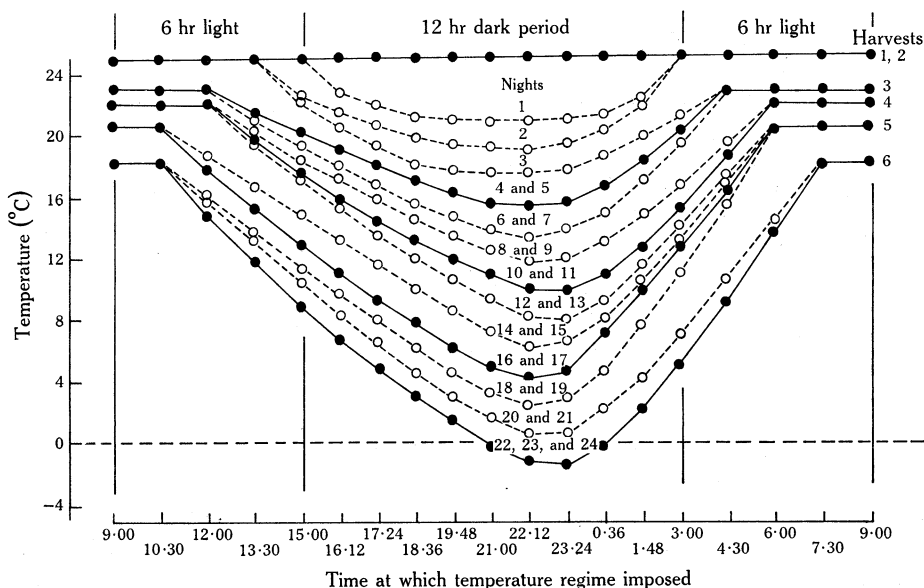


Fig. 1.—Temperature regimes imposed for 24 days of chilling treatment involving gradual departure from a constant 25°C.

As in the previous trial (Gates *et al.* 1973) the pots were shielded against changes in air temperature by insertion through an insulating lid of foam plastic into a large tank of water, under thermostatic control. It was thus possible to maintain a temperature difference between tops and roots.

The experimental layout was the two species \times two root temperature regimes \times six harvests at differing degrees of cold stress \times three replicates, in factorial design.

Harvests were at days 38, 45, 50, 56, 62, and 69 from sowing. Frosting conditions were simulated, with the increasingly cold nights and warm bright days that are normally experienced in the autumn in south-east Queensland. A 12-hr day with an air temperature of 25°C was imposed at harvest 1, and a gradually increasing measure of cold stress from harvest 2 to harvest 6 as shown in Figure 1. The amount of cold stress imposed based on the departure from 25°C totalled: harvests 1 and 2, nil; harvest 3, 320 deg-hr; harvest 4, 1190 deg-hr; harvest 5, 2580 deg-hr; harvest 6, 4990 deg-hr departure from a constant 25°C (approximating to a quadratic curve of increase). Root temperature regimes are presented in Figure 2 where "normal" is the regime of the previous trial (Gates *et al.* 1973), and "chilled" is lower than it by some 10°C, with the aim of impairing the development of the two species differentially. These root temperature regimes may be compared with typical curves (Fig. 2) for minimum soil temperatures at a depth of 15 cm at 9 a.m., and for minimum grass temperatures for recurrent frostings during July 1961, at Samford, south-eastern Queensland.

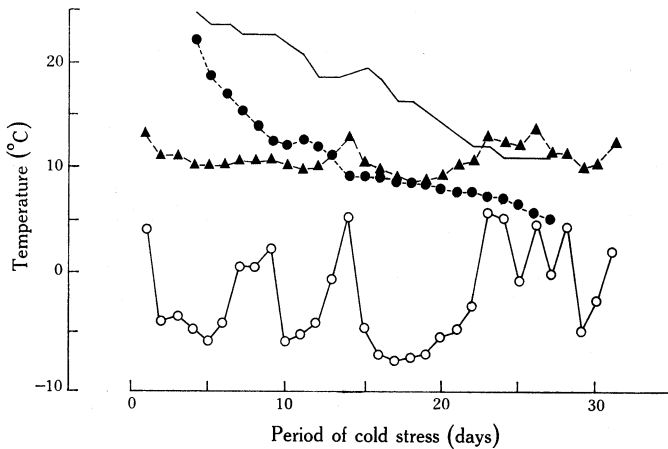


Fig. 2.—Curves showing minimum grass temperature in field at 9 a.m. (○), soil temperature at a depth of 15 cm in field (▲) at Samford, Brisbane, July 1961, and the gradually reducing temperatures to which the pots were exposed (●) as cold stress of a similar intensity to the field was imposed. — Normal root temperature in pot.

At harvest, fresh weight of tops was recorded, and also dry weights of tops and roots after drying under forced draught at 80°C. Nitrogen and phosphorus were estimated on the ground tops and root samples by autoanalysis of Kjeldahl digests with selenium catalyst after the method of Williams and Twine (1967). Replicates were bulked for analysis.

(b) Numerical Analyses

Previous studies in this laboratory (Gates *et al.* 1970, 1973) have suggested strongly that, in interspecific studies of response to stress, little interest resides in any individual variate. Although small differences between species for individual variates can be detected, the overall comparison is more meaningful when considered as an integrated set of such small differences. The ideal approach would therefore be to examine the configuration of all variates simultaneously. Since this is impossible for more than three variates, the problem is primarily one of reduction of dimensionality: to find a small number of optimum linear combinations of the original variates which together account for most of the variance in the system. Such an approach is not aimed at testing a hypothesis, but at obtaining an economical description. In these circumstances the function of classical statistical analyses is that of a preliminary check, to safeguard the experimenter against the possibility that any patterns extracted are due simply to experimental error. The numerical analyses thus fall into three sections:

(i) *Preliminary Statistical Appraisal*

For the replicated set of data, eight possible variates appeared to merit attention. These were dry weights (in grams) of tops, roots, and whole plants; fresh weight (g), water content (g), and percentage water in tops; and dry-weight ratios of root to tops and to whole plants. For the unreplicated set, 21 variates were considered: fresh weight (g) and water content (g) in tops; dry weight (g) and nitrogen (mg), phosphorus (mg), their ratios, and percentage nitrogen and phosphorus in tops, roots, and whole plants; milli-equivalents of phosphorus per gram of water in tops. Since economical description is an essentially multivariate process, and since many of these variates are highly correlated, a multivariate analysis of variance would be desirable; however, the unreplicated set is so nearly over-defined and some of the correlations necessarily so high that the error dispersion matrix must be almost singular. The calculation of Wilks' criterion would thus be ill-conditioned, and such an analysis would be unreliable. The only alternative was to carry out a complete set of univariate analyses. This would normally be regarded as invalid, since it is tantamount to assuming that the variates can be treated as independent, which they are not; but this objection will not apply if the sole intention of the procedure is to establish that the system contains sufficient variation, not attributable to error, for further examination.

(ii) *Classificatory Study*

In most forms of dimensionality reduction, difficulties in interpretation of vectors after the first may be encountered if the system contains marked discontinuities. It was therefore decided to select a subset of variates and subject the 24 treatments \times species \times harvests to a classification based on these variates. The subset chosen (from the unreplicated set) comprised fresh weight (g) (mean per pot); dry weight (g), nitrogen (mg) and phosphorus (mg) content, and percentage nitrogen and phosphorus in tops and roots; and the root to whole plant ratios for dry weight, nitrogen, and phosphorus, 14 attributes in all. Since all the attributes were continuous variables, a Euclidean model was appropriate; that chosen was the model of Burr (1968), which, although the computational route is different, effectively scales all variates to equal sample variance. The fusion strategy was the same author's "incremental sum of squares" (Burr 1970—sometimes known as "Ward's error sum of squares"), which minimizes the within-group sums of squares on fusion.

(iii) *Ordination Study*

For dimensionality reduction the standard procedure would be principal components analysis. However, since the main interest resided in the configuration of the 24 elements, it was convenient to use its *Q*-analogue, or *dual*, principal co-ordinate analysis (Gower 1966, 1967). Both the classification above and the Euclidean distances needed for the Gower procedure were obtained by the use of options on the program MULTCLAS, available on the Control Data 3600 computer in Canberra.

III. RESULTS

(a) *Visual Appraisal*

At normal root temperatures, treatment responses were apparent visually as early as at 1000 deg-hr of cold (just prior to harvest 4), when the leaves began to show purplish coloration in *S. humilis* but were quite healthy in *L. bainesii*. By harvest 5 (2700 deg-hr of cold) these differences were marked, the leaves becoming a brownish yellow in the former, especially on the more exposed parts, but remaining healthy in the latter. *S. humilis* had originally transpired more water than *L. bainesii* but this relation was reversed to 20 v. 120 ml per day. Development appeared to have ceased in *S. humilis* at harvest 5, but was relatively unimpaired in *L. bainesii*. At harvest 6 (4900 deg-hr of cold) some frost burn to leaves on the ends of exposed shoots was apparent in both species, and the dormant state of *S. humilis* plants was quite evident.

At chilling root temperatures, differences between the species were initiated earlier and became more marked. *S. humilis* ceased growing and reduced its tran-

spiration before 1000 deg-hr of cold (harvests 3 and 4). Thereafter, it quickly deteriorated, the leaves became very brown, and some dehisced. *L. bainesii* appeared to cease growing and to harden under the combined effects of cold to root and tops. It thus was less susceptible to frost injury, the leaves merely deepening in anthocyanin coloration.

TABLE 1
NUMBER COMPARISONS ATTAINING THE STATED LEVEL OF SIGNIFICANCE

	Replicated set (8)			Unreplicated set (21)			Total
	$P = 0.05$	$P = 0.01$	$P = 0.001$	$P = 0.05$	$P = 0.01$	$P = 0.001$	
Temperature (T)	—	—	7	3	8	4	22
Species (S)	—	—	8	—	8	9	25
Harvest (H)	—	—	8	—	5	15	28
T × S	1	2	—	2	1	—	6
T × H	—	1	3	3	—	3	8
S × H	—	—	6	9	1	2	18

(b) Preliminary Statistical Appraisal

The level of significance attained by the main effects and single interactions is summarized in Table 1. It will be noted that the main effects are almost everywhere significant; of the interactions, only species × harvests is consistently significant. Nevertheless, of the 174 possible comparisons, 107, or 61 %, were significant, so that there is no danger that any patterns elicited by numerical methods will reflect only experimental error.

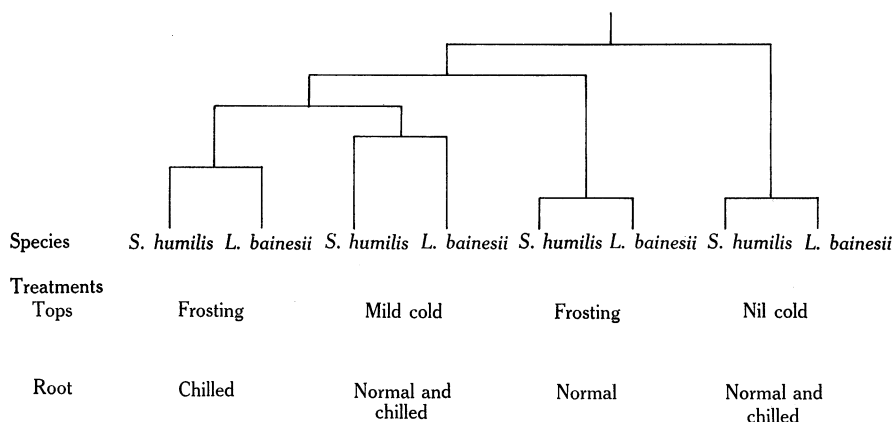


Fig. 3.—Hierarchy for multivariate classification of weight and nutrient composition in tops and root of *S. humilis* and *L. bainesii*.

(c) Classificatory Study

Figure 3 presents the hierarchy for the classification of weight and nutrient responses in tops and roots. Although the procedure is agglomerative, it will be convenient to adopt the convention of reading the hierarchy downwards, and referring to the fusions as "divisions".

The chief subdivision in the classification is between the initial favourable growing temperature regime and subsequent chilling. This, of course, is confounded with plant size, so that size attributes were responsible for this contrast. At the next subdivision of the hierarchy, plants of the frosting sequence (2500 and more degree-hours of cold) under normal root temperature regime separated from others due to the favourable effects of normal root temperatures on development of the roots. Root dry weight, amount of nitrogen and phosphorus in the roots, and the ratios of these root attributes to tops were all higher for plants under frosting conditions at normal root temperatures. Under mild cold, normal and chilled root temperatures did not differ detectably. At chilling root temperatures the frosting series differed from mild cold in having lower nutrient content in their tops.

At the final subdivision of the hierarchy a distinction exists between the two species. At nil cold this resulted from tops size differences, but at 2500+ deg-hr of cold root attributes made a notable contribution, which will be considered later.

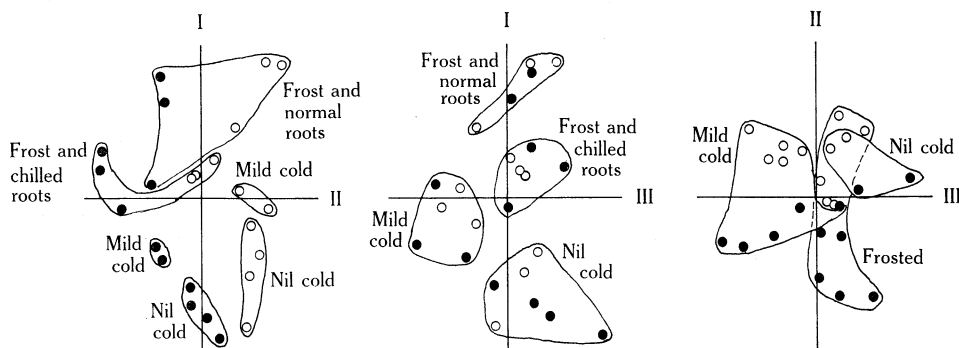


Fig. 4.—Principal components for yield and nutrient composition in tops and root of *L. bainesii* (●) and *S. humilis* (○).

(d) Ordination Study

Figure 4 presents the principal co-ordinates for the 24 attributes representing species \times root temperatures \times harvests combinations. These separated into clearly defined patterns on the first vector of the analysis. This vector accounted for 69% of the variance, largely because of plant size differences. As the plants grew, absolute amounts rose and concentrations fell as occurs in most growth experiments. The size differences were in this case confounded with cold treatment, for when subjected to greater amounts of cold, development was slowed, as is demonstrated for chilled roots versus normal in the frosting series.

The two species separated along the second vector although they still showed similar age and cold treatment responses. The nitrogen concentration of the tops and roots, the root weight ratio, and the fresh weight of the tops were the principal attributes contributing to this, but the trends in nitrogen concentration were negative for roots and positive for tops. This suggests a species difference in response to cold stress whereby one species ceased to grow and fix nitrogen in its roots whilst the other continued. Trends in fresh weight were also negative, indicating a greater degree of leaf injury and subsequent dessication in one species than the other under cold stress.

The nature of this relation may now be clarified by a re-examination of species differences at the final level of the classification in Figure 3.

TABLE 2

MEAN VALUES FOR PRINCIPAL ATTRIBUTE CONTRIBUTIONS TO THE FROSTING SEQUENCES IN *L. BAINESII* AND *S. HUMILIS*

Attribute	Normal root temperatures			Attribute	Chilled root temperatures		
	<i>S. humilis</i>	<i>L. bainesii</i>	Attribute contribution		<i>S. humilis</i>	<i>L. bainesii</i>	Attribute contribution
Root weight ratio	0.3	0.2	0.22	Tops nitrogen (%)	3.5	2.2	0.26
Root dry weight	7.7	4.7	0.15	Root weight ratio	0.2	0.1	0.12
Tops fresh weight	62.3	90.2	0.14	Root phosphorous (%)	0.2	0.3	0.10
Root nitrogen (%)	1.8	2.8	0.12	Tops nitrogen (mg)	44.7	32.5	0.08
Tops nitrogen (%)	3.7	3.2	0.03	Tops fresh weight	54.3	72.1	0.08
Root phosphorous (%)	0.1	0.2	0.02	Root nitrogen (%)	2.6	3.3	0.07
Total contribution			0.74				0.89

Table 2 indicates that it was *S. humilis* which ceased development, for its attribute means for root nitrogen and fresh weight were some 25% less than *L. bainesii*; whilst percentage nitrogen of its tops was some 50% higher. The value for the tops indicates a check to development since nutrient concentrations fell with time in developing plants of this trial.

The third vector contributed only 9% to the total variance, so that each of the other two vectors may be considered in relative isolation when graphed against vector III as in Figure 4. When this was done, plant size differences were dominant on vector I and species did not separate. Vectors II and III, on the other hand, showed distinct species separation, thus confirming the species difference within the series subjected to frost. Separation on the basis of cold, as distinct from plant size was also evident. Although not indicated on Figure 4, within each species the effects of normal root temperatures were less severe than chilled, for their positions on vector II were more positive than for chilled roots.

The time trends for multivariate response to cold stress are presented in Figure 5 for vectors I and II which together account for 83% of total variance. Vector I confirms the check to development received by both species in response to cold. It also shows the greater magnitude of the effect in the case of *S. humilis* and its enhancement by root chilling. Vector II, being free of the confounding effect of plant size, shows these cold effects even more clearly.

It has been seen that some attributes (Table 2) made a notable contribution to the net response to cold depicted in Figures 3 and 4. These smaller responses which interact to comprise the resultant difference between species, are worthy of enumeration. The attributes which will now be briefly discussed are: ratio of root to tops dry weight, fresh weight of tops, and relative nitrogen content of tops.

For these specific attributes it is appropriate to return to the original univariate analyses of variance. Table 3 gives the ratios of dry weight of root to dry weight of tops. The interaction of species \times root temperature and of harvest \times root temper-

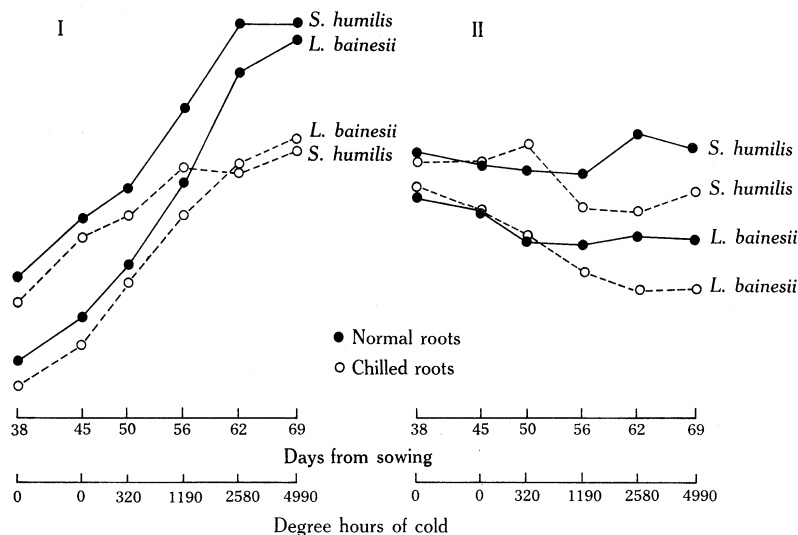


Fig. 5.—Trends for increasing cold stress in first (I) and second (II) principal coordinates for yield and nutrient composition.

ature were highly significant ($P < 0.01$ and $P < 0.001$, respectively). The favourable effects of normal root temperatures which were noted in the numerical analysis are thus confirmed. Figure 6 presents relative water content of plant tops. Species \times

TABLE 3
CONTRASTS IN ROOT TO TOPS DRY WEIGHT RATIOS FOR INCREASING COLD STRESS DURING DEVELOPMENT IN *L. BAINESII* AND *S. HUMILIS*

Harvest	<i>L. bainesii</i>		<i>S. humilis</i>	
	Normal root temperature	Chilled	Normal root temperature	Chilled
1	0.158	0.174	0.252	0.202
2	0.152	0.148	0.250	0.212
3	0.113	0.120	0.239	0.209
4	0.167	0.126	0.302	0.215
5	0.274	0.150	0.500	0.210
6	0.292	0.146	0.483	0.252

harvest interaction was highly significant ($P < 0.01$) and so also was the interaction of root temperature \times harvest ($P < 0.001$). As cold increased, water content in both species decreased, as might be expected for a gradual increase in cold. This would allow the plant time to harden. Levitt (1956) concluded that the hardening process

involves a reduction in the percentage of water. The degree of change was greatest for *L. bainesii*, especially when the roots were chilled, for its water content decreased further. Table 4 presents the changes in relative nitrogen in the tops, confirming significant species ($P < 0.01$), harvests ($P < 0.001$), and root temperature ($P < 0.05$) differences, without interaction.

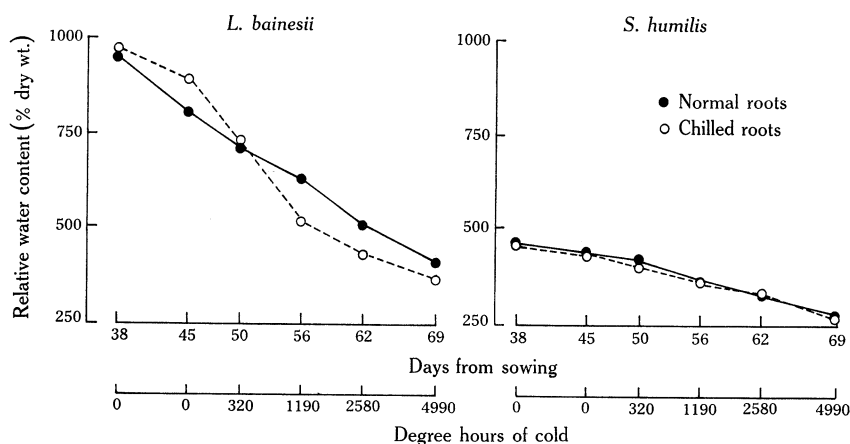


Fig. 6.—Relative water content of plant tops.

The major contributing attributes thus confirm the overall response assessed by the numerical approach, and they confirm the greater measure of cold tolerance possessed by *L. bainesii* when compared with *S. humilis*.

TABLE 4
RELATIVE NITROGEN CONTENT OF PLANT TOPS
Values as percentage dry weight

Harvest	Degree-hours of cold	<i>L. bainesii</i>		<i>S. humilis</i>	
		Normal	Chilled	Normal	Chilled
1	0	4.3	4.7	4.9	4.9
2	0	4.5	4.5	4.6	4.9
3	320	4.2	4.1	4.4	4.2
4	1190	3.4	3.1	3.7	3.5
5	2580	3.3	2.2	3.7	3.4
6	4990	3.0	2.2	3.7	3.4

IV. DISCUSSION

As shown previously (Gates *et al.* 1966, 1973) the age of the plants and phosphorus level in the present study were both suitable for normal growth and nitrogen fixation in both *L. bainesii* and *S. humilis*. It was also shown that this combination suited *L. bainesii* under cold stress, although studies under only chilling and drought conditions have been made with *S. humilis* (Gates *et al.* 1971).

When subjected to cold stress, both legumes suffered a pronounced check to growth. This is shown by a rapid and highly significant drop in relative growth rates to almost nil in both species as cold increased (Table 5). Species also differed with

TABLE 5
WHOLE PLANT RELATIVE GROWTH RATES
Values given as grams per 100 g per day and show a significant main effect for species, $P < 0.001$, and a significant main effect for harvest intervals, $P < 0.001$

Harvest interval	Degree-hours of cold	<i>L. bainesii</i>		<i>S. humilis</i>	
		Normal	Chilled	Normal	Chilled
1-2	Nil	6.9	12.0	7.0	7.8
2-3	0-320	11.7	12.5	5.0	3.6
3-4	320-1190	5.5	7.1	6.0	3.4
4-5	1190-2580	5.9	3.2	4.2	-0.4
5-6	2580-4990	2.1	3.1	-0.7	2.1

high significance. Although it is quite normal for relative growth rates to fall with time, they do not fall so markedly under normal growing conditions. Figure 5 confirms this check to development, and shows the greater magnitude of the effect in *S. humilis*, especially with root chilling. The response was also apparent from visual observation. The estimation of overall plant response indicated that *S. humilis* was the first to cease development, and this was confirmed by the discrete responses to cold that were selected by the numerical analysis as making a notable contribution to the net response. The greater measure of cold tolerance possessed by *L. bainesii* relative to *S. humilis* is thus confirmed from a number of aspects, both qualitative and quantitative.

Gates *et al.* (1973) indicated that *L. bainesii* could withstand an extensive amount of cold stress, its productivity and nitrogen fixation being favoured during stress by high levels of phosphorus fertilization. This was most successful with mature plants. These conditions were supplied here, along with the added stress of root chilling. Whilst *S. humilis* was checked in development by merely atmospheric cold, *L. bainesii* could undergo frosting temperatures with only minimal frost injury and was hardened still further by the added influence of root chilling. Previously, it was determined that although phosphorus-treated plants were less affected by chilling temperatures, they were more susceptible to frosting injury, apparently because of enhanced tissue nitrogen levels and succulence. In the present case, chilling of the roots led to less frost injury and lower tissue water levels than with normal temperatures. The conclusion reached by Levitt (1956) may well apply here that "the greater the degree of hardiness developed the greater is this reduction" in percentage tissue water. Visual observation confirmed the lower frosting injury for root-chilled plants of *L. bainesii*.

These features of cold tolerance are a valuable propensity for a tropical legume, and warrant the fullest exploitation both in practice and by further scientific evaluation. In practice it may be possible to exploit the capacities of species of the genus *Lotononis* more. A large number of species in the genus occur extensively throughout

southern Africa. For a genus whose normal growth is favoured by a tropical temperature regime, many of these species are uniquely tolerant of cold, and some of them are promising pasture legumes (Strickland, personal communication). The genus warrants close practical study. However, most studies of tolerance to cold have involved such a sudden change from normal growing conditions to chilling or frosting conditions, that the plants received a cold shock quite unlike that to which they are exposed in nature. Under natural conditions the day-length changes associated with the march of seasons are accompanied by varying degrees of temperature change, and these influences may accrue to harden the plant in autumn. Bryan (1972) imposed a sudden shock upon *L. bainesii* for the specific purpose of checking growth to a maximum degree, and he recognized its difference from natural conditions. It is therefore desirable when assessing cold response as an agronomic issue to impose a catenary sequence of temperature and day-length change that does not cause a cold shock more severe than that usually experienced in the field. This has seldom been achieved, although it was the basis of the present studies.

The scientific evaluation of response to cold stress should include metabolic features. This has been initiated for *S. humilis* by Gates *et al.* (1971), who followed changes in time between amino acids, and soluble and residual nitrogen in relation to dry weight. The analysis of metabolic features by multivariate techniques proved of considerable value in that study in the appraisal of the important role of proline relative to other amino acids, under stress conditions. The methods are a logical extension from practical studies to metabolic comparisons that seek the dominant features of plant response through time. There have been no metabolic studies of *Lotononis* species, and there have been few interspecific comparisons in response to cold, perhaps because they may be expected to be complex, as mentioned in the Introduction.

Norris (1965) has suggested that legumes which have specialized rhizobial requirements also may be expected to have specialized physiological adaptations. *L. bainesii* is highly specialized in its rhizobial affinities whereas *S. humilis* is not. There is real need to study the metabolic aspects of cold tolerance in these two species to determine whether *L. bainesii* owes its adaptability to special metabolic features or to a greater stability of enzymatic behaviour than exists in *S. humilis* when constrained by cold from usual growth patterns.

Unfortunately, metabolic studies of cold response under frosting conditions have often been with plants held in the dark for periods of several days. The present series (Gates *et al.* 1971, 1973) and that of Bryan (1972) have been for plants treated under adequate light at favourable day length (Gates 1970). This facility is essential for successful frosting studies, as has been indicated by Olien (1967) who states the hardening process involves metabolic activity, so that the plant must have access to light or an energy reserve. Unfortunately, there are no relevant species comparisons for cold stress studies of this type.

V. ACKNOWLEDGMENTS

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