Genetic and Environmental Control of Flowering in Trifolium repens in the Tropics

Abstract. Trifolium repens at low elevations expressed wide genetic variation in tendency to flower. Clones classified as flowering or nonflowering were subjected to temperatures associated with high elevations. Flowering in "nonflowering" clones was induced under warm-day- cool-night treatments. It is proposed that in the tropics, low temperatures associated with high elevations are an important factor in determining flowering, and therefore ability to persist, in plants which are long-day and temperature sensitive.

The role of climate in controlling vegetative and reproductive phases of plant development is a major factor in plant distribution (*1*) as well as of immediate concern to plant producers. Many plants are grown in a climate quite different from that where the seed was produced. *Trifolium repens* has long been grown in Hawaii, where it flourishes in higher elevations. As it has been considered a long-day plant, its flowering ability poses a problem, since tropical days do not attain the length of those in the temperate zone. Low night temperatures are known to promote flowering in a number of long-day plants (*2*). Roberts and Struckmeyer (*3*) tested *T. repens* as one of some 120 species and varieties of plants. Under Wisconsin greenhouse conditions, flowering in the species was demonstrated by cold-night treatment. Quantitative studies were desirable on this point, particularly as they relate to field conditions. The role of genetic factors also needed clarification.

Field collections in Hawaii of *T. repens* showed wide genetic diversity in flowering tendency. Clones at Honolulu (elevation 70 feet) manifested all degrees of flowering from none to profuse. Field experiments with plants grown at different elevations had suggested that temperature under Hawaiian conditions did have a profound effect on flowering. Experiments were therefore planned to shed light on this problem by the use of a controlled environment cabinet. The cabinet was designed to produce diurnal fluctuations of temperature as well as any desired length of day. The "day" temperature was set at 65°±3°F, and the "night" at 47°±3°F, the temperatures found at a high elevation station in the field experiments. A 13-hour day length was chosen because this approached the maximum long day found at Honolulu.

Two clones, A and B, which flowered under Honolulu conditions, and three which did not, C, D, and E, were chosen. Sufficient cuttings of each clone, for four treatments each with six replicates, were grown in vermiculite. They were watered three times a week with Hoagland's solution and the other days with tap water.

The four treatments were as follows. The first group was grown outdoors under Honolulu conditions subject to the summer temperature of Honolulu (13 June—30 Sept.). Because of Honolulu's maritime climate, variation was not great. The mean maximum temperature was 83.1° and the range was 77° to 88°F. The mean minimum was 72.3° and the range was 63° to 77°F. This treatment group was subject to warm days and warm nights. The second treatment group remained in the cabinet and thus was subject to cool days and nights. A third group was moved to outdoors for 8 hours during the day and then into the cabinet at night so that it was subject to warm days and cool nights. The fourth group was placed in the cabinet for 8 hours each day and then moved outdoors. It was thus subject to cool days and warm nights.

Results are shown in Fig. 1. The data show that the "nonflowering" clones, C, D, and E, produced flowers when subjected to the warm-day and cool-night treatment (third group). No other treatment was successful in inducing flowering in C, D, and E. Flowering in the clones A and B was reduced by a cool-day, warm-night treatment. (Vegetative growth was also reduced in this particular treatment.)

The experiment was repeated, omitting treatment 4 (cold-day, warm-night) and clone D. The same results were obtained, that is, the warm day, cool-night treatment produced flowers in the "nonflowering" clones.

The work shows that flowering in the "nonflowering" clones is promoted by subjecting them to cold night temperatures, even though they were exposed to the warm days of Honolulu. It is therefore apparent that the low night temperature associated with the high elevation station is the probable mechanism for triggering flowering in these clones. This work is in agreement with that found by Hiesey for *Poa* (*4*).

It should also be emphasized that certain clones (A, B) flowered freely without being subject to cold nights, indicating that flowering in this species may be considered a phenotypic response of the genotype to its environment, just as are morphological characters. As with some morphological characters, there is apparent wide genetic diversity within the species.

The failure to flower of clones C, D, and E in the cabinet (cold-day, cold-night) was unexpected. Two possibilities exist. The first is that the light intensity in the cabinet was not sufficient to produce good flowering. The second is that low night temperatures are not the stimulating agent for flowering, but rather the change in temperature.

The results are of practical value because the seed of many forage crops is produced in areas where environmental conditions are quite different from the area where the crop itself is to be produced. If a crop consists of a mixture of phenotypes, the environmental conditions of the area in which the seed is produced may tend to favor some phenotypes over others by virtue of differential flowering. Therefore, an un-

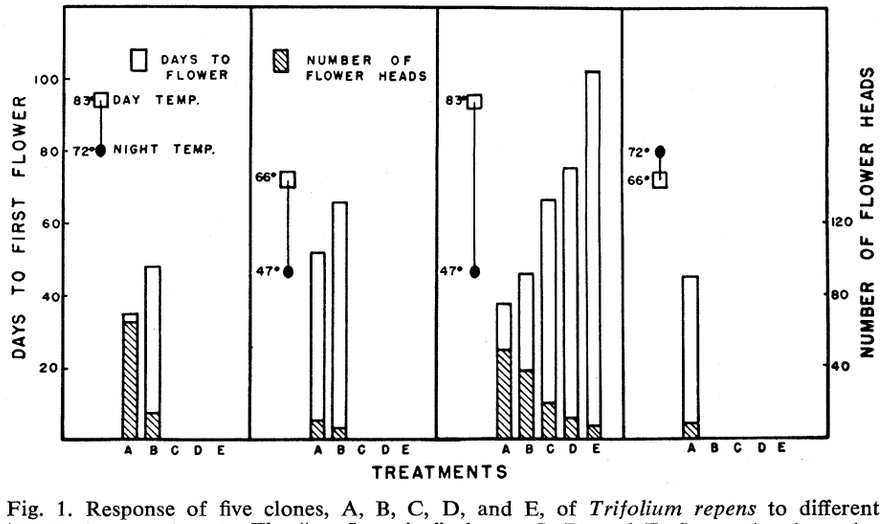

Fig. 1. Response of five clones, A, B, C, D, and E, of *Trifolium repens* to different temperature treatments. The "nonflowering" clones, C, D, and E, flowered only under warm-day and cool-night treatment. Clear bars represent days to first flower (mean of six replicates). Shaded bars represent number of flower heads (total of six replicates).

conscious selection may be carried on.

The findings present an interesting picture of reproduction of long-day, temperature zone plants introduced to the tropics. It is shown that low night temperatures, or changes of temperature associated with high elevations, constitute a mechanism for inducing flowering. The ability of such plants to persist is, therefore, apparent (*5*).

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Mammalian Liver β-Glucuronidase for Hydrolysis of Steroidal Conjugates

Abstract. Although the rate of hydrolysis by mammalian β-glucuronidase appears to be inhibited by methylene chloride or carbon tetrachloride with the standard technique (phenolphthalein glucuronide as a substrate), the release of steroidal conjugates under conditions generally employed does not appear to be affected.

Gautney *et al.* (*1*) have recently described the potentiating effect of several organic solvents (aromatic hydrocarbons, chlorinated aliphatic hydrocarbons, aliphatic alcohols) on bacterial β-glucuronidase, thus extending an earlier report concerning chloroform (*2*). The authors, however, found that dichloromethane and chloroform inhibit the hydrolysis of phenolphthalein glucuronide by mammalian liver enzyme. This raises the question whether mammalian liver β-glucuronidase is suitable for hydrolysis of steroidal glucuronides in biological fluids which have previously been extracted with such solvents.

Table 1. Effect of solvents on hydrolysis by liver β-glucuronidase of various glucuronides.

Enzyme units/ml	Control (No solvent)	Dichloromethane (% Aglycon)	Carbon Tetrachloride (% Aglycon)
	Phenolphthalein		
6.25	2.2	1.0	2.8
12.5	5.4	2.2	4.9
25.0	15.0	4.7	12.7
50.0	32.0	9.7	30.0
250.0	69.0	54.0	71.5
1000.0	100.0	98.0	100.0
	17-Ketosteroids		
10.0	16.0	9.4	10.7
100.0	45.2	43.3	41.0
500.0	100.0	96.0	94.0
	Corticoids		
10.0	16.0	14.0	13.3
100.0	58.5	67.5	62.3
250.0	85.5	81.3	81.0
500.0	100.0	99.5	100.0

Previous studies in our laboratory had demonstrated quantitative release of the free steroid when pregnane-3α, 17α, 21-triol-11, 20-dione monoglucuronide, added to human plasma previously extracted with dichloromethane, was incubated with 300 to 500 Fishman units of mammalian β-glucuronidase per milliliter (*3*). In view of the implications of Gautney's (*1*) results, this matter was reinvestigated.

A pool of human urine was divided into aliquots of 10 ml, and duplicates were equilibrated with either dichloromethane or carbon tetrachloride. Thereafter the urine was incubated with beef liver β-glucuronidase (*4*) in doses of 10 to 500 Fishman units per milliliter at *p*H 4.5, 37°C for 48 hours. The 17-ketosteroids were extracted and measured as reported elsewhere (*5*), and the urinary corticoids were determined by the method of Silber and Porter (*6*). In addition, the mammalian enzyme was studied by use of phenolphthalein glucuronide as substrate after the manner of Talalay *et al.* (*7*), with the addition of 0.1 ml of the organic solvent to replicate digestion mixtures employed by Gautney (*1*) in his investigation.

The results (Table 1) are expressed as percentage of maximal hydrolysis achieved with each substrate. In agreement with Gautney *et al.* (*1*), the solvents inhibited hydrolysis of phenolphthalein glucuronide by the low concentrations of enzyme often employed. On the other hand, under the conditions of incubation with high enzyme concentrations for 48 hours, as generally described for hydrolysis of steroid conjugates (*3, 8*), no significant inhibition was apparent. At lower concentration of enzyme the solvents only slightly reduced the partial hydrolysis of steroid glucuronides. These results do not support the assertion by Gautney *et al.* (*1*) that such solvents must not be used for prior extraction if liver β-glucuronidase is to be employed for subsequent hydrolysis of steroid glucuronides.

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Glucuronidase Activation: Enzyme Action at an Interface

Abstract. The potentiating action of chloroform on bacterial β-glucuronidase has been shown to increase as the interface area between the two liquid phases increases. Prior extraction of the enzyme with chloroform causes a loss rather than an increase in activity. It is tentatively suggested that the correlation between activity and interface area may reflect a phenomenon of enzyme action at a liquid/liquid interface.

In an investigation of the "paradoxical" effect of organic solvents on the activity of animal and bacterial β-glucuronidase, which was first observed with chloroform (*1*), and more recently with other solvents by Gautney, Barker, and Hill (*2*), we have also observed an activation of the bacterial enzyme (*3*) varying in degree with the solvent used and a similar inactivation of the animal enzyme (*4*). We have further noted that the activation of the bacterial enzyme seems not to be due to the removal of an inhibitor, since prior extraction of the enzyme solution by shaking with one volume of chloroform for 5 minutes causes a 40 percent loss rather than an increase in activity. An activation such as that noted by Bernfeld, Jacobsen, and Bernfeld (*5*) has been ruled out by the work of Gautney, Barker, and Hill. Moreover, the continuous removal of phenolphthalein from the reaction site cannot account for the inverse effect on the animal and bacterial enzymes. These facts suggest that some physicochemical property of these enzymes is involved in the activation by solvents. The preliminary results reported here relate to this problem.

The assay method used in this study is that suggested by the Sigma Chemical