Table 1. Effect of Pretreatment on Mortality of Wheat Seedlings after Freezing

Pretreatment	Mortality		
Oxygen (%) / Temperature (° C)	0–1	2–3	21
0·0	35·1 *	24·1	13·2
12–15	33·0	46·2	60·7

Difference for significance $P = 0{\cdot}05$ 16·0
$0{\cdot}01$ 22·0

* Transformed value ($P = \sin^2\theta$) of percentage mortality, 3 weeks after freezing.

Reduction of oxygen supply had some protective effect, but was also inhibitory to the normal process of low-temperature hardening. The beneficial result was similar in magnitude to treatments with hydrocyanic acid, narcotics and reduced moisture supply, all of which were capable of bringing about hardening, but not so effectively as did low temperature. These results are in accord with findings of other workers[2] and may best be attributed to the influence of reduced growth rate, which is common to all treatments. In no case, however, has an effect of the magnitude observed by Siegel *et al.* been reported.

It is suggested that a likely explanation of their results is that treated plants failed to freeze. In short-term experiments super-cooling is readily achieved and, in fact, usually has to be guarded against by artificial inoculation. Differences in the moisture content of the atmosphere in which plants were frozen could well contribute to differences in behaviour in this regard.

Experiments reported here were carried out with facilities provided by the University of Sydney.

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[1] Siegel, S. M., Halpern, L. A., Guimarro, C., Renwick, G., and Davis, G., *Nature*, **197**, 329 (1963).
[2] Levitt, J., *Frost Killing and Hardiness of Plants. A Critical Review* (Burgess Pub. Co. : Minneapolis, Minn., 1945).

Polysiphonia urceolata in Axenic Culture

Efforts to obtain axenic cultures of red algae hitherto have been successful only with unicellular species[1,2] or multicellular species with monosiphonous thalli[3,4].

Among the unialgal cultures maintained in this laboratory there are species with more complicated structures such as *Ceramium strictum*, *Lomentaria clavellosa* and *Lolysiphonia urceolata*. Small pieces of these species were treated with penicillin in a solution containing 3,000 I.U./ml. After 24 h the species were placed on agar plates with only 2,000 I.U./ml. for 10 days. The pieces of *Lomentaria* and *Ceramium* had then lost their colour, whereas *Polysiphonia* appeared alive. The pieces of this alga were then transferred to agar plates containing nutrient medium *ASP* 6*F* with 5 g ion-agar, 5 g glucose, 1 g asparagine and 0·5 g '*N-Z*-case' (an enzyme-hydrolysed casein : Sheffield Chemical), per litre[5]. Most pieces were overgrown by bacteria or yeast in a few days, but around three pieces no infections could be observed even after 14 days. These small branches were then transferred to flasks containing 25 ml. of the artificial seawater *ASP* 6*F*, where they grew out. Later, small pieces from these cultures were tested for purity, and in December 1962 it was possible to establish that *Polysiphonia urceolata* had been obtained in axenic culture.

As other multicellular red algae had turned out to be more or less vitamin-heterotrophic, the requirement for growth substances was investigated. It was difficult to obtain a suitable form of inoculum for experiments, as *Polysiphonia* grows with only slightly ramified branches and does not form any sort of spores in culture. Tips of branches were used as inocula, and it was found to be necessary to use branches with at least two or three lateral branches, if the alga were to grow out in all flasks of a series.

The alga could then be tested for possible vitamin requirements. In order to produce as good a response as possible, the alga was starved for vitamins through cultivation in vitamin-free *ASP* 6*F* and transferred into a new solution every week. After four weeks an experiment was performed in the following series : (*a*) with no vitamins ; (*b*) with the complete vitamin solution of *ASP* 6*F* ; (*c*) with B_{12}, riboflavin, pyridoxamine and folic acid ; (*d*) with the remaining 11 substances of the vitamin solution. Growth was very variable within the series, because too small inocula had been used in this experiment ; but in series (*a*) and (*d*) no growth at all occurred. The vitamin demand was absolute as the vitamin-starved alga died after cultivation in a vitamin-free solution for 8 weeks. *Polysiphonia* is thus vitamin-heterotrophic and the necessary growth substance or substances were among vitamin B_{12}, riboflavin, pyridoxamine and folic acid. As the earlier examined red algae, *Goniotrichum elegans*[3] and *Erythrotrichia carnea*[6], were B_{12}-heterotrophic, this compound might be suspected to be the active substance.

In the next experiment, B_{12} was tested alone or combined with other vitamins. As Table 1 shows, B_{12} was necessary for growth of *Polysiphonia*.

Table 1. Growth of *Polysiphonia urceolata* with Vitamin B_{12} (= Cyanocobalamin) Alone or in Combination with Various Other Vitamins. Incubation Time, 36 days. Inocula starved for 28 days

Vitamin added in the same amount as in *ASP* 6*F*	Dry weight of algae from 6 flasks (mg)
No addition	2·0
All vitamins	23·6
B_{12}	22·6
B_{12}, riboflavin, folic acid and pyridoxamine	26·6
B_{12} and riboflavin	30·8
B_{12} and folic acid	28·0
B_{12} and pyridoxamine	26·4

Table 2. Growth of *Polysiphonia urceolata* with Different Naturally Occurring B_{12} Analogues. Incubation Time, 30 days. Inocula starved for 26 days

B_{12} analogues (1 μg/l.)	Base of nucleotide	Dry weight of algae from 6 flasks (mg)
No addition		1·8 *
Cyanocobalamin (= B_{12})	5-6-dimethylbenzimidazole	24·0
Factor III	5-hydroxybenzimidazole	20·4
Factor I_b = Factor *B* phosphoribose	None	11·0
Factor *B*	None	5·0 *
Pseudovitamin B_{12}	Adenine	8·8 *
Factor *A*	2-methyladenine	4·8 *
Factor X_1	None	13·4
Factor Z_1	None	16·6

* The alga died before the end of the experiment.

Different, naturally occurring B_{12} analogues were also tested (Table 2). During the first part of the experiment the alga grew with all additions. The best growth was obtained with cyanocobalamin and Factor III, but *Polysiphonia* did not seem to 'prefer' Factor III to vitamin B_{12} as does *Goniotrichum*[7]. Another difference in the utilization of the different analogues could also be observed. *Goniotrichum* is unable to use Factor *B* or pseudovitamin B_{12}, whereas Factor *A* gives as good growth as cyanocobalamin. In the series where these three factors were added, growth ceased after some days, and at the end of the experiment the algae were dead. This might indicate that factors with another base than benzimidazole in the nucleotide, or with no nucleotide at all, could be used only for some metabolic purposes.

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[1] Koch, W., *Arch. Mikrobiol.*, **18**, 232 (1953).
[2] Giraud, G. (personal communication).
[3] Fries, L., *Nature*, **183**, 558 (1959).
[4] Fries, L., *Experientia*, **17**, 75 (1961).
[5] Fries, L., *Physiol. Plantar.*, **16**, 695 (1963).
[6] Fries, L. (unpublished results).
[7] Fries, L., *Physiol. Plantar.*, **13**, 264 (1960).