THE ASEPTIC CULTURE OF *ARABIDOPSIS THALIANA* (L.) HEYNH.

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[*Manuscript received January* 11, 1957]

Summary

Arabidopsis thaliana (L.) Heynh. is a small, rapidly growing crucifer with a 28-day life cycle, a low chromosome number ($n=5$), and a high fertility (152 ± 11 seeds under aseptic conditions). The small size and rapid growth of the plants enable them to be grown throughout their life cycles on sterile, nutrient agar in ordinary test tubes. Alternatively, up to 50 plants may be grown on silica gel in crystallizing dishes. For optimal growth the trace element cations should be chelated and light should be excluded from the roots. When supplied with sucrose the plants absorb only the glucose portion. During growth the plants excrete small quantities of vitamins and other substances into the medium but not in amounts likely to prevent the detection of growth mutants through cross-feeding.

I. INTRODUCTION

The methods described below for the culture of *Arabidopsis thaliana* (L.) Heynh. were developed as a necessary preliminary to the study of the physiological genetics of flowering plants. For such work, the conditions of culture should meet two requirements. Firstly, the medium should be nutritionally inert except for those inorganic elements or radicals necessary for plant growth; and secondly, plants should be maintained in an aseptic condition throughout their life cycles.

The detection of mutants which occur with low frequency even after mutagenic treatment requires, especially with a diploid organism, that a relatively large number of plants be grown under closely defined conditions. However, the methods of aseptic culture that have previously been published are unsuitable for the present purposes, because in practice they allow the culture of only one or a few plants and these for usually only a part of their life cycle.

There appear to be only two published reports of a phanerogam completing its life cycle under completely sterile cultural conditions. The first of these (Laibach 1943) states that *A. thaliana* may be grown in test tubes on nutrient agar from germination to seed ripening, but no details are given. The second account (Loo 1946) concerns the culture of the desert ephemeral, *Baeria chrysostoma* T. & G. Seeds of *Baeria* were germinated on filter paper and the seedlings were transferred to an agar or sand medium in 300 by 25 mm tubes. The plants flowered after about 30 days and set some seed which, however, had low viability (less than 10 per cent. germination).

It is evident that successful aseptic culture on an adequate scale is mainly dependent on the nature of the experimental plant. Considerations of space, generation time, and genetic suitability require a plant of small size, rapid growth, low chromosome number, and high fertility. Such a plant, *A. thaliana*, was described by Laibach (1943) who provided the initial seed stocks.

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II. Material

A. thaliana (*Arabis thaliana* L.) belongs to the tribe Arabideae of the Cruciferae. The plant occurs throughout Europe, East Africa, and Northern and Central Asia. A large number of ecotypes, differing chiefly in photoperiod requirement, has been collected and examined by Laibach (1943, 1951).

The seeds when properly stored, retain their full ability to germinate for about 3 years (Laibach, unpublished data). During the present studies it was found that seeds, completely after-ripened, germinated in 2 days at a temperature of 22–26°C and with at least 16 hr of light per day. Such germination also occurred when seeds were taken from the siliqua 12 days after fertilization. Thereafter, the seeds entered upon a dormant period which lasted for about a fortnight after full-ripening. This after-ripening dormancy could be broken by subjecting the seeds to a temperature of 2°C for 8 hr followed by 24°C for 16 hr, and then repeating the cycle. Fully after-ripened seeds usually gave 94–99 per cent. germination.

The photoperiodic response of *A. thaliana* has been examined by Gregory and Hussey (1953). They found that, given 8 hr of daylight each day together with supplementary artificial illumination, the number of days to flowering was progressively reduced the longer the total daily period of light. A further progressive acceleration of flowering was obtained with an increase in the intensity of the supplementary light up to 50 f.c.

When grown in soil, *A. thaliana* may reach a height of 15–35 cm depending upon the ecotype. According to Salisbury (1942) the average seed output of plants growing in the wild is 1650 $\pm$ 150. Plants grown in aseptic culture are much smaller, and their reproductive capacity is lowered accordingly (152 $\pm$ 11 seeds per plant). The plant is fully self-fertile, and all ecotypes examined were found to possess a haploid chromosome number of five.

Seed of 10 "races", named after the localities from which the seed was originally collected, were planted under the standard conditions described below in order to select races most suitable for genetical experiments. Of these, the race Enkheim possessed the most rapid development, requiring 13·6 $\pm$ 0·3 days from germination to flowering. However, the race Estland which requires 21·9 $\pm$ 0·3 days to flower was finally selected because of its vigorous uniform growth.

III. Methods and Results

(*a*) *Cultural Conditions*

Major mineral requirements were provided as Knop solution and, except for an increased quantity (0·002M) of KH_2PO_4, at the concentrations recommended by Arnon (1938). The trace elements supplied were iron, manganese, zinc, copper, boron, and molybdenum: iron at 2 p.p.m. and the remainder as used by Arnon (1938). The chemicals were of A.R. grade and the water was glass-distilled.

The mineral solution was adjusted to pH 6·0 approaching the lower ecological pH of *A. thaliana* which, according to Small (1946), is 6·2. During growth, the pH of the medium increased to about 7·1. However, a stable pH obtained by balancing the partial concentration of nitrate against that of ammonia (Trelease and Trelease 1933) did not give significantly better growth.

The nutrient solution was solidified with 0·75 per cent. agar previously purified by extraction with ethanol and pyridine. Besides providing a support for the growing plant, agar is said to make oxygen more accessible (Rippel and Lehmann 1936) and thus the aeration required in liquid cultures is here avoided. The agar concentration is important, for if the gel is not firm enough the leaves become saturated with water and distorted growth results. If the agar is too firm, however, there is variation between plants in the penetration of their primary roots and these delays are carried throughout their subsequent development.

The melted medium was dispensed in 5-ml aliquots to 16 by 150 mm "Pyrex" test tubes, which were then plugged with non-absorbent cotton wool and autoclaved at 15 lb pressure for 15 min. Sometimes the seed production may be much reduced and this effect can generally be attributed to pollen abortion because of an inefficient gas exchange to the outer atmosphere; cotton-wool plugs, therefore, should be quite loose.

The seeds were sterilized by immersion in a solution of absolute ethanol and 20 volumes hydrogen peroxide (1 : 1) for 10 min (Harris, unpublished data), and planted with a platinum loop inside a sterilized cabinet. Seeds may be transferred from this sterilizing solution directly to the agar without there being any subsequent toxic effects. When planting, the seeds should be laid on the surface of the agar; if they are even slightly below the surface the plant may fail to orient itself properly.

Immediately after planting, the cultures were placed at a low temperature (0–5°C) for 24 hr to ensure uniform germination. They were then transferred to a growth chamber which was maintained at 25°C and supplied with continuous illumination of about 800 f.c. intensity from fluorescent light tubes. It was found advantageous to keep the relative humidity above 60 per cent. to prevent the agar medium from drying out too rapidly.

A satisfactory indication of growth rate was obtained by noting the daily increase in length of the primary root, the number of secondary roots, and the date of appearance of successive leaves, floral initials, and the first open flower. In Figures 1, 3, 4, and 5 the mean values and standard deviations (shown by vertical lines) are based on 12–15 plants per treatment.

(*b*) *Factors Affecting Growth*

(i) *Trace Elements.*—The medium first used consisted only of an aqueous solution of major and trace elements solidified with agar. The plants on this medium, however, rapidly became chlorotic, showed little root growth or leaf expansion, and usually died before setting seed. The general appearance of the plants suggested a trace element deficiency which was confirmed by the finding that the plants were bright green and grew vigorously with the addition of a solution of Seitz-filtered trace elements. Increasing the concentration of iron from 1 to 2 p.p.m. ensured better growth and survival but there was still marked chlorosis during vegetative growth, although the leaves turned green at the time of flowering. Supplying the iron in a chelated form as ferric citrate gave no improvement over ferric sulphate; the mean dry weight at flowering with ferric citrate was $6{\cdot}0 \pm 0{\cdot}3$ mg, and with ferric sulphate, $6{\cdot}4 \pm 0{\cdot}3$ mg.

When ethylenediaminetetra-acetate (EDTA) was used (according to the method of Jacobson 1951) to chelate the ions of copper, zinc, and manganese as well as iron, growth was equal to that obtained with Seitz-filtered trace elements (Fig. 1).

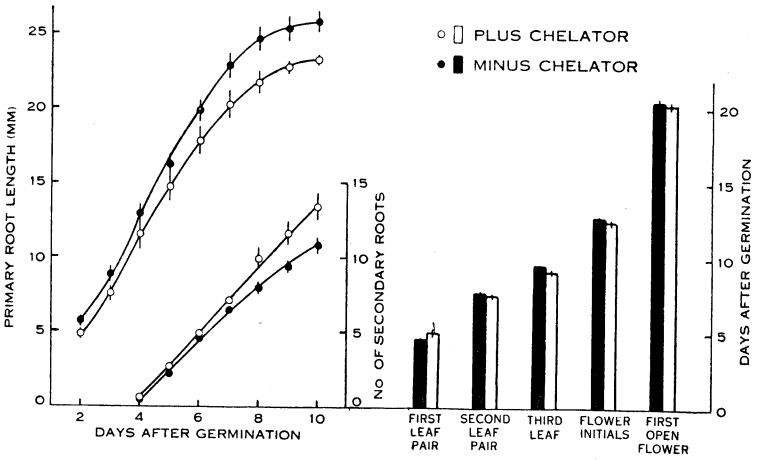

Fig. 1.—Effect of trace element chelation on growth.

Chelation with EDTA resulted in greater secondary root formation, increased vegetative development, and increased dry weight: with EDTA, $8{\cdot}1 \pm 0{\cdot}3$ mg, and without EDTA, $6{\cdot}4 \pm 0{\cdot}3$ mg.

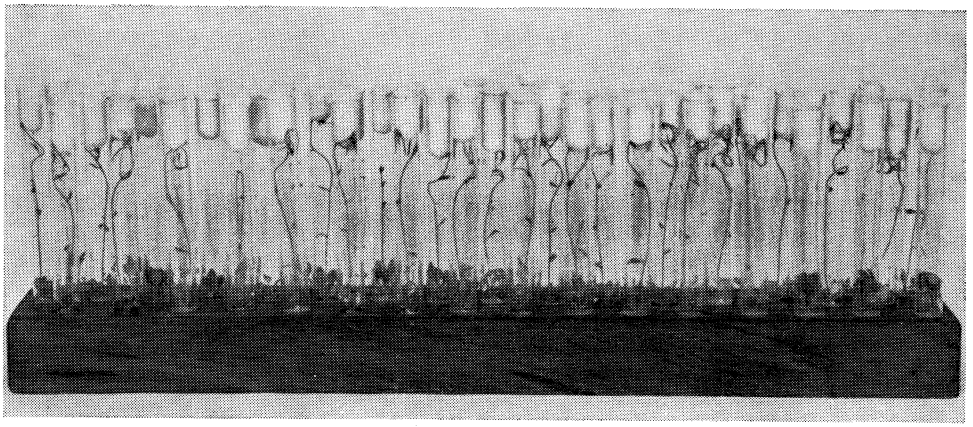

Fig. 2.—Wooden block holding test-tube cultures of 48 plants, 26 days old.

(ii) *Exposure of the Root System to Light.*—In order to obtain optimal growth it was found necessary to shield the root systems of the growing plants from direct light. This was best accomplished by placing the test tubes in holes bored in wooden blocks so that the surface of the agar medium was level with that of the wood (Fig. 2).

The primary effect of light upon root growth was a general decrease in meristematic activity as reflected in shorter primary and fewer secondary roots

(Fig. 3). This inhibition of root growth in turn delayed the formation of floral primordia (by 1 day) and the onset of flowering (by 1·5 days), and also significantly decreased the total dry weight at flowering from the normal 8·1±0·3 mg to 6·9± 0·5 mg.

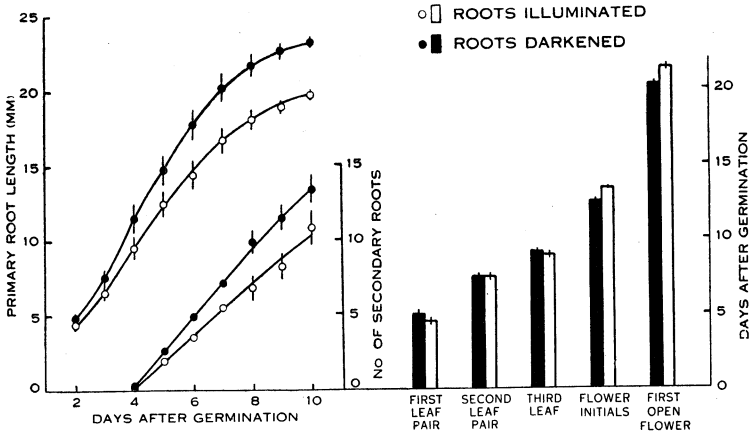

Fig. 3.—Effect on growth of exposing the root system to light.

(iii) *Sucrose.*—As the intensity of light available to the plants is less than the 800 f.c. reaching the test tubes, it was thought advisable to supplement the photosynthetic supply of carbohydrate. To determine the most readily utilizable sugar, a chlorophyll-deficient mutant, which possessed otherwise normal chloroplasts, was supplied with a range of carbohydrates usually at the rate of 100 mg/plant.

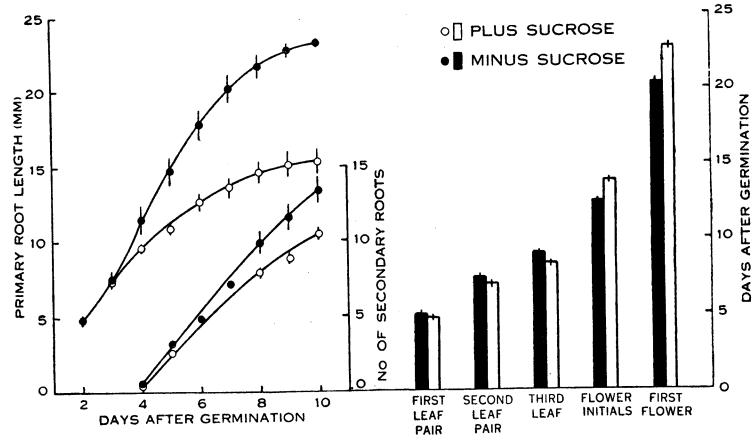

Fig. 4.—Effect of 2 per cent. sucrose on growth.

Sucrose gave the best growth, with glucose only slightly inferior; fructose was a very much poorer carbon source. Although glucose was utilized, there was no growth with glucose phosphates or with other phosphorylated sugars. Sucrose, purified by boiling with activated charcoal, significantly accelerated vegetative development (Fig. 4) and increased the plant mean dry weight from 8·1±0·3 mg to 9·4±0·2 mg.

At the same time, however, it depressed root growth and delayed flowering by 2·5 days.

After the plants had finished growing, the agar from 50 test tubes was examined for substances excreted by the plants during growth. This agar contained large quantities of a carbohydrate which was identified by the melting point of its osazone and by its chromatographic behaviour as fructose. Further chromatographic analysis of the agar at intervals during plant growth showed that although the fructose

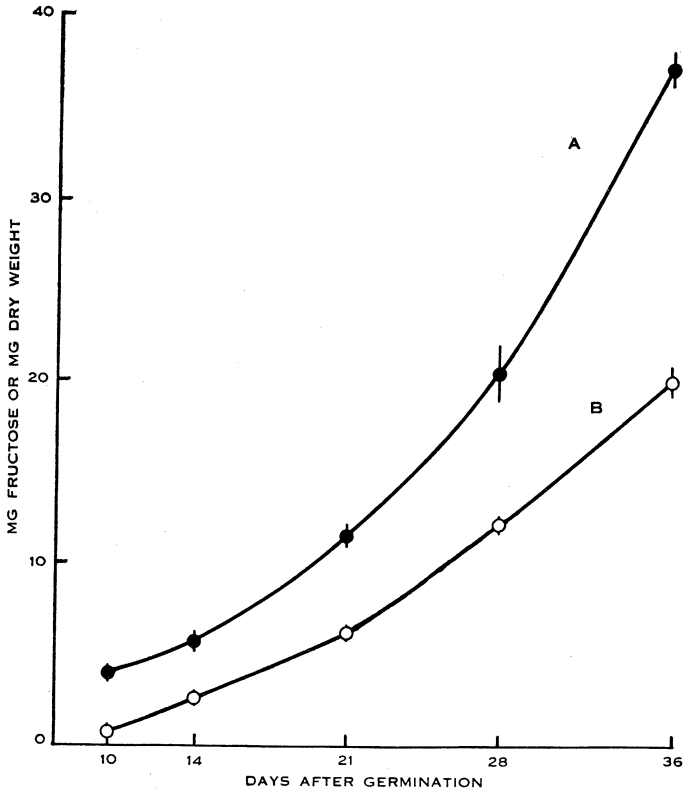

Fig. 5.—Rate of fructose formation (or glucose uptake) in mg/plant (*A*), and the corresponding plant dry weights (*B*).

increased in amount, no glucose was to be found. It was concluded that the plants had hydrolysed the sucrose provided in the medium, absorbed the glucose, and left the fructose behind.

The rate of glucose uptake was then followed by quantitative determination of the residual fructose by the Hagedorn-Jensen method for reducing sugar. The curves for fructose production, which may be taken as representing glucose uptake, and the corresponding dry weights of the whole plants are shown in Figure 5. At the time of seed formation each plant was absorbing about 2 mg of glucose per day.

(c) *Large-scale Culture*

In a segregating progeny only one or two plants in 14 are mutants because of the chimerical nature of the parent plants which develop from irradiated seed, and thus the isolation of mutants by individual plant culture is quite laborious. Therefore, attention was turned to finding methods of growing the plants in bulk culture.

Agar is unsuitable as a medium for large-scale culture because it must be darkened with charcoal to protect the plant roots from light and this leads to heating of the medium and excessive water loss from the agar surface. Many other substrates were tested, and two of these, cellulose pulp and silica gel, proved to be reasonably satisfactory. A cellulose pulp medium is the more easily prepared, but it is readily metabolized by fungi, and different samples of what is supposed to be the same grade of cellulose pulp differ widely in their suitability for plant growth. Silica gel, on the other hand, may be prepared in such a way as to give a standard product, and contamination with microorganisms is not a serious problem.

The method of silica gel preparation was adapted from the work of Daiber (unpublished data, 1944). The silicic acid sol is prepared as follows: 12·5 ml of liquid sodium silicate is diluted with 8·4 ml of glass-distilled water. This solution is added slowly and with cooling to 31 ml of concentrated hydrochloric acid (sp. gr. 1·18). It is necessary to use distilled water, for impurities may cause premature opalescence or coagulation of the sol during dialysis. The silicic acid sol must be dialysed to remove sodium chloride and excess acid and the dialysis should be carried out at a temperature below about 16°C to avoid gel formation. The solution was electrodialysed, using a current of 10 mA, in a cold room for about 24 hr. By this time the resistance of the solution had increased from less than 1 ohm to a constant value of about 50 ohms. 75 ml of the dialysed sol was then mixed with 40 ml of triple-strength mineral nutrient solution, the pH adjusted to 6·0, and 75 ml of the sol was placed in each crystallizing dish (9 by 5 cm). The solution gels in about 1 hr at room temperature, the rate of gelling depending on the pH, salt content, and age of the sol. The dishes and contents were sterilized by steaming for 30 min and planted with sterilized seed in the usual manner. Fifty seeds may be planted in each of 12 dishes in 1 hr.

To keep the cultures aseptic, the space about the lid of the crystallizing dish was packed with cotton wool pre-treated with cetyl pyridinium chloride (0·1 per cent. chloroform solution). The inner surfaces of the lids may be treated with "Anti-dim" to prevent water condensing in large drops and reducing the available light. When the flower stalks reached the lids the plants were about to flower and the lids were then removed to allow flowering and seed-setting to take place normally. Fungal contamination at this stage of the plants' growth did not affect their further development.

Growth was more variable on silica gel than on agar mainly because of the greater occurrence of delayed root penetration with the former substrate. This disadvantage, however, was offset by the much larger populations which could be screened for mutants.

(*d*) *The Excretion of Organic Substances*

A possible objection to the use of bulk-culture methods for the isolation of growth mutants is that cross-feeding between normal and mutant plants may occur, thus preventing the detection of certain types of mutants. Accordingly, a study was made of the excretions from the roots of wild-type *A. thaliana* when grown in agar.

Plants were grown to maturity on agar medium to which 2 per cent. purified sucrose had been added on the assumption that a high carbohydrate level in the plant might increase the amount of excretion. All plant material was removed from the agar which was then examined for vitamins, amino acids, and reducing substances.

The vitamin assays were carried out using vitamin-requiring mutants of *Neurospora crassa*. The agar samples to be tested were inoculated in duplicate with conidia from a given mutant and incubated at 24°C for 5 days. Wild-type *Neurospora* grew well on aliquots of the agar with no additions, this indicating the presence of biotin. Using suitable mutants, qualitative tests were made for eight vitamins of the B group, but activity was found only with respect to pyridoxine, thiamine, and inositol. Pyridoxine and inositol were present in amounts suboptimal for maximum growth of the fungus as shown by a yellowing of the medium on which the pyridoxine-less mutant was grown, and by the colonial growth of the inositol-less mutant.

α-Amino nitrogen in the agar was estimated colorimetrically using ninhydrin, by the method described by Milton and Waters (1949). Although this method is sensitive to 5 μg of amino nitrogen per ml, no positive reaction was obtained.

The agar was next examined by extracting with ethanol, chromatographing the extracts, and spraying the chromatograms for reducing substances. Only fructose was found, and, after mild acid hydrolysis, fructose and glucose from the residual sucrose were found.

A thin layer of white material appeared on the surface of the agar near the plant stem at about the time the plants flowered. This wax-like substance was very insoluble and was not further examined. As the plants grew, the medium showed a blue fluorescence in ultraviolet light which deepened as growth continued. However, all attempts to isolate the fluorescent substance were unsuccessful.

These tests appear to indicate that wild-type plants do not excrete sufficient material to interfere with the detection of growth mutants when segregating plants are grown together.

IV. Discussion

A small rapidly growing plant such as *A. thaliana* offers many advantages for experimental work on flowering plants. It is especially suitable for mutation and other genetic studies because of its short life cycle, few chromosomes, and high seed production. These advantages are enhanced by the relative ease with which

plants may be maintained under sterile conditions. The consequent precision of environmental control facilitates physiological experiments on growth and development, the addition of organic substances to the substrate, and the study of gene–environment interactions. In addition, the small seeds with their low nutrient reserves make the plants very sensitive to mineral deficiencies, and thus potentially valuable for mineral bioassay. A brief report of the use of *A. thaliana* in the production and isolation of biochemical mutations has recently appeared (Langridge 1955).

The critical factors for optimal growth in aseptic culture are chelation of trace element cations and protection of the root system from direct light. Chelation is presumably necessary to avoid precipitation of cations during autoclaving of the medium. The continued growth of excised tomato roots in culture has similarly been shown to be limited by an unavailability of iron which may be overcome by the addition of chelated iron (Street, McGonagle, and McGregor 1952). According to Weinstein, Robbins, and Perkins (1954) the complexed iron is not only more readily absorbed by plant roots, but is also maintained in a form which is more readily metabolized by the plant.

The mode of action of light in inhibiting root meristem activity, with a consequent lowering of total dry weight, is not known with certainty. It has been shown by Naundorf (1940) and Pilet (1953) that light causes changes in the auxin concentration of roots. However, the former author found that illuminated roots of *Helianthus* produced more auxin than non-illuminated ones, while the latter stated that light caused an inactivation of auxin in *Lens* roots. Although indolylacetic acid was supplied in a range of concentrations to light-exposed roots of *A. thaliana*, it failed to increase root growth or dry weight.

The ability of *A. thaliana* to utilize different sugars is similar to that reported for other plants (e.g. Allsopp 1953). Sucrose and glucose are about equal in value as carbon sources, fructose is only slowly utilized, and most other sugars are either quite inferior or toxic. Dormer and Street (1949) have found that excised tomato roots will grow on sucrose, but not on glucose, fructose, or an equimolecular mixture of the two. They suggest that this is because carbohydrate can only be absorbed as hexose phosphate and that hydrolysis of sucrose is necessary to provide the energy for phosphorylation. Although roots of *A. thaliana* can hydrolyse sucrose either by a mechanism operating at the root surface or by the secretion of an enzyme into the substrate, there is no evidence that energy available from hydrolysis is used in phosphorylation of the glucose. The fact that free glucose supports as good a growth of albino mutants as does sucrose suggests that external phosphorylation is not essential for absorption by the intact plant.

V. Acknowledgments

I wish to thank Professor D. G. Catcheside, formerly Professor of Genetics, University of Adelaide, for his helpful advice; Professor Emeritus F. Laibach of Frankfurt-on-Main for seeds of *A. thaliana*; Mr. C. G. Greenham, Division of Plant Industry, C.S.I.R.O., for help with the electrodialysis; and the Commonwealth Scientific and Industrial Research Organization for the award of a studentship.

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