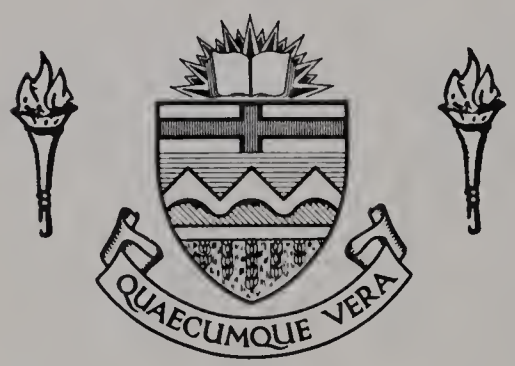

THE UNIVERSITY OF ALBERTA

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"Some aspects of the physiology and ecology of the chromosome races of Viola adunca J.E.Smith"

by

© George Davis

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Some aspects of the physiology and ecology of the chromosome races of Viola adunca J.E.Smith" submitted by George Davis in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in ECOPHYSIOLOGY.

To the people of South Africa committed

to the realization of a just society.

Abstract.

It is observed in many northern hemisphere plant species that polyploids tend to have a wider, and often more northern distribution than diploids. An explanation which has been offered, suggests that either the polyploids are more tolerant of environmental extremes, or that they are better able to recolonize disturbed sites viz. those glaciated during the Pleistocene. Viola adunca, a perennial herb indigenous to North America and represented by a diploid and a tetraploid race, was subjected to eco-physiological scrutiny in an attempt to find the basis for its two-part distribution. Plant material was collected in an area of sympatry in southeastern Alberta.

Results of the controlled environment experiments conducted here appear to suggest that the cold hardiness of mature plant leaf tissue, whole seedlings, and incipiently germinating seeds, is the same for both the diploid and the tetraploid. Tests indicated that hardened leaf tissue could withstand temperatures less than -20C, hardened seedlings temperatures less than -16,C and cold-treated imbibed seeds temperatures less than -20C. The diploid mature plant however, which often displays a more compact growth form, may be able to withstand slightly more severe cold stressing for short periods on account of a reduced susceptibility to heat loss. A second exception appeared in unhardened seedlings, where diploids suffered a 47% mortality at a

stress temperature of -6C, but tetraploids none. On the other hand, seed production, which occurs primarily by way of cleistogamous flowers in both races, is distinctly more prolific in the case of the tetraploid. The same is true of the germinability of those tetraploid seeds, and of the net productivity of the arising seedlings under favourable growing conditions. The root:shoot ratio of the tetraploid seedlings was also significantly higher at 57 days than that of the diploids (.40 as opposed to .34). The alternative mode of regeneration, whereby shoot tissue arises from the root stock of the previous year's growth, appears to be more rapid (occurring about 10days earlier) in the case of the diploid, a phenomenon interpreted here as a type of entrenchment behaviour. A consideration of the possibility that rhizospheric associations with nitrogen-fixing microorganisms might be assisting the colonization process on the nutrient-poor glacial till, led to a brief investigation via the acetylene-reduction assay. The results obtained confirmed the existence of such associations, but failed to attribute them solely to either one or the other chromosome race. Soil moisture is thought to have an effect on the degree of activity of the fixers, which included Clostridium spp.

It is suggested from the synthesis of the data gathered that the apparent success of the tetraploid race of V. adunca in the glaciated regions of North America can be attributed to its superior propagational ability, whereas

its reverse spread into the undisturbed parts of the continent has been prevented by the tenacity with which the diploid occupies its existing niche.

Preface.

In March 1977, this project was officially launched under the financial motive force of the Boreal Institute Grant BAR 5530019 to Dr. J.M.Mayo. In September of that year, having been persuaded through circumstance to set aside temporarily my fancied obsession with xerophytic water relations, I stood as a neophyte on the periphery of cytotaxonomic phytogeography, assured only by my confidence in Dr. Mayo as guide and mentor.

What evolved can be described as an exciting and eclectic personal journey in space, time, and the other dimensions which define Botany as a discipline. The result is a type of sketch in physiological eco-taxonomy. (I trust that the nebulousness of this designation sets it enough apart so as not to be construed as a trespass on areas more formally delineated). This journey however, has not been without its troubles: times lost, like Bunyan's pilgrim, in mires of uncertainty and sloughs of despond. But once committed to an objective search, such trials are inevitable, as epitomised by the Zen story dealing with learning:

> Hakuin used to tell his pupils about an old woman who had a teashop, praising her understanding of Zen. The pupils refused to believe what he told them and would go to the teashop to find out for themselves.

> Whenever the woman saw them coming she could tell at once whether they had come for tea or to look into her grasp of Zen. In the former case, she would serve them graciously. In the latter, she would beckon them to come behind her screen. The instant they obeyed, she would strike them with a fire-poker.
>
> Nine out of ten of them could not escape her beating[1].

To be content with an intuitive grasp of the truth (drinking the tea without overt enquiry), is the more serene option, but the nature of objective science forces us to submit ourselves to the pain inflicted by the poker, perhaps to enjoy with a fuller understanding the cup of tea at a later date. And so for me, Viola adunca will eventually return to its real but obscure role in what we arbitrarily call the North American Flora, but hopefully, from this work, a part of its essence will have been extracted for future use by interested parties.

In this academic extraction process, I have received help from many quarters, direct and indirect. The set of names mentioned below by no means constitutes a complete list, but it does indicate the main supports without which this phase of the characterization of Viola adunca could not have happened. I would like to thank Dr. Mayo for his key role in providing information, inspiration, moral support

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[1] Reps, P. (compiler). Zen Flesh, Zen Bones. Pelican. 1971.

and the financial wherewithal; Rudy Kroon, Hermann Barthel and Bev Taylor for propagating and perpetuating (and putting up with the weedy ubiquitousness of this species); Dr. Doug Whitfield for initially encouraging me to investigate the avenue of Botanical academia; and Dr's. Bliss and Keith Denford for being helpful advisors on my thesis committee. Other people to whom I owe thanks are: Ann Stebner and Dr. Cook of Soil Science for checking the identity of possible nitrogen-fixers associated with Viola's roots; Phil Wright and John Konkin for their patience and skill in dealing with not-always-cooperative growth cabinets; John Harter, Bill Russell and Pat Seymore of this department, and Andrew Pierce of the Denver Botanic Gardens for the living plant material from locations beyond the scope of the project's budget; Scott Russell for his help with the photographic plate making; Dr. Pawan Bassi of Plant Science for checking the ethylene standards used in the gas chromatography; Elfie Doerrbecker for total cooperation in the borrowing and lending of this and that; and the many students and staff members who helped to create an atmosphere of stimulation, good humour and philosophical flexibility.

And then there are the personal acquaintances who deserve my thanks. They are the close friends who were made to endure erratic hours and the presence of the daemon Viola in their midst, and especially Stacey, who bearded the computer to help in getting the bulk of this text committed

to its binary gut. And lastly but foremostly there are my parents, who once searched the Karoo for Mesembs.

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I. Introduction.

In the study of the evolution of the angiosperms, an important role has been attributed by many authors to the phenomenon of polyploidy, which occurs at a frequency of approximately 30% in the dicotyledonous species of this plant group, and even higher in the monocots.(Löve, 1964). G.L.Stebbins, one of the foremost proponents of this viewpoint, has suggested that extra replicates of the basic genome tend to increase the ecological amplitude of the possessor (1971). This he bases on the observed geographical distributions of some well known genera and species represented by both diploid and polyploid sub-taxa, which generally exhibit a wider distributional range in the case of the polyploid, often encompassing sites disturbed by the Pleistocene glaciation.

Strict autoploidy, judging from the results obtained for artificial polyploidization (Ellerström and Hagberg, 1954; Stebbins, 1976), tend to be limiting with regard to fitness, and it would appear safe to assume that at least some degree of hybridization (segmental alloploidy) is necessary during formation under natural conditions, for the manifestation of adaptive advantage in polyploids. The main advantage of the multiplicity of genomes is thought to lie in the ability of the plant to take advantage of new gene-combinations when the need arises, while maintaining the functions adapted to the existing environment (de Wet, 1971).

In order to consolidate our understanding however, and to clarify our ideas on polyploidy as an evolutionary mechanism, much experimental work will be needed to bridge the gap between the set of observations offered by the cytologists, and that which describes geographical distributions. This in other words, points to critical experimental work within the broad spectrum of plant physiology, from gene-related enzyme studies, to general ecological characterizations of plant function in its natural environment.

One of the first suggestions that linked ploidy level with adaptive ability, was that of Hagerup (1931), who proposed that there existed a positive correlation between the frequency of polyploidy and environmental severity. Subsequent work by various researchers (see Johnson et al., 1965, for a synoptic review) helped to develop the idea that an observed increase in the frequency of polyploidy with latitude could be attributed to the superiority of polyploids in tolerating environmental extremes (e.g. Tischler, 1935; Löve and Löve, 1957). Johnson and Packer (1965), in a survey of the angiosperm flora of the Ogotoruk Creek area in northwestern Alaska, show a correlation between the frequency of polyploidy and edaphic environmental severity, which coincides on a local scale to the global model based on Hagerup's work. They reported that the frequency of polyploidy was 88% at one extreme of the environmental gradient (soils with fine texture, high

moisture, low temperatures, shallow permafrost and high disturbance), while at the other it was only 43% (on soils with characteristics antithetical to those listed above). Another observation arising from the Ogotoruk Creek study, is the fact that the overall polyploid frequency for that flora is lower than for any area of equivalent latitude in Europe. The authors attribute the latter to the fact that the area was not subjected to glaciation during the Pleistocene, and that the relatively high frequency of diploids is a relict reflection of the Tertiary flora.

Observations such as these have pointed to the complexity of the relationships between ploidy levels and their environments, and to remove the narrow focus from the role of hardiness per se. In their review, Johnson et al. (1965) suggest the alternative hypothesis to explain the northern spread of polyploidy, which is that polyploidy confers advantages on angiosperms that allow for an improved ability to colonize the areas laid waste by the Pleistocene glaciations, or that such an ability might be acting in conjunction with improved tolerance to environmental extremes.

Briggs and Walters (1969) point out that another feature of polyploidy is the frequency with which it is associated with apomixis. They suggest that the high percentage of polyploids in the higher latitudes is perhaps nothing more than a reflection, on a whole flora level, of their greater tendency towards apomixis, an adaptation well

suited to the colonization of disturbed sites. (This does not seem to be of importance in Viola adunca, as vegetative reproduction is not thought to be a major propagational strategy with respect to colonization in either race).

Much of the work referred to above has, owing to a fundamental lack of data, a somewhat scholastic air about it. Statements regarding tolerance of extremes, colonizing ability, and reproductive modes, are all inferred from cytogeographical observations based for the most part on whole floras, and species composition is not generally taken into account. The data required for the development of more precise and more compelling hypotheses than those so far available to us, must necessarily help elucidate the causal links between cytological and distributional observations. That is, more information regarding the mediating physiological processes is needed.

A strong bias in biological research of the past few decades has directed much effort towards attempts at understanding the complex relationships between the organism and its environment. Refinements of approach in this discipline of ecology have led to the establishment of the now well entrenched sub-discipline of physiological ecology, a tool well adapted to developing an understanding of the chain of events linking the two poles currently of interest to us. A fruitful, though by no means exhaustive approach has been to conduct comparative investigations into the behaviour of closely related taxa, differentiable primarily

at the ploidy level. And even now, when insight into a limited number of species and situations has been afforded by experimental research, it is evident that a simple unifying hypothesis may well be undermined by the large number of exceptions, and that each species, subspecies, or race, can only be accounted for in terms of its own peculiar autecological strategies.

Two relatively recent studies can be used to illustrate this last point. The first, by Hall (1972), discloses that the roots of autopolyploid rye seedlings require more oxygen for normal respiratory activity than do diploid seedlings. This is characterized by the critical temperatures for root apical meristem activity, which are 22C and 15C for the diploid and the tetraploid respectively. This might suggest that warmer soil temperatures are less easily tolerated by the polyploid. Jackson (1976), in his review of polyploidy, suggests that this work may point to a reason for the distribution of the diploids and polyploids observed by Johnson and Packer (1965) in the Ogotoruk Creek flora mentioned above.

A second study, also dealing with graminoid seedlings, is that of Tyler et al. (1978), who showed a complex pattern of seed and seedling ecology in the chromosome races of Festuca pratensis. The tetraploid F. pratensis var. apennina, which is found only in mountain grasslands above 1500m, was found to produce seeds which required a cold treatment in order to germinate. The diploid of the lowland

grasslands, on the other hand, could be propagated via seeds not exposed to a cold treatment. When germinated seedlings were tested for cold hardiness after an acclimation period, it was found that the tetraploids were far more susceptible to cold than the diploids. The authors proceeded to propose that the two ploidy levels exist within their respective ranges by virtue of different strategies, that is, the diploid is able to outcompete the tetraploid at lower altitudes by the late-season annexation of space, coupled with an ability of its seedlings to survive the milder winter temperatures, whereas the tetraploid has adapted to avoid the lethal winter temperatures of the higher altitudes by adopting a strategy of spring germination.

These two studies, in their vastly different expressions of the same phenomenon of polyploidy, serve to accentuate the danger in holding too rigidly to the belief that there might exist a unifying theory to explain the differential distribution of chromosome races of the same species. The notion that we may be able to generalize with any precision about the distribution of different species (and higher taxa) with respect to ploidy, also becomes more remote with the acceptance of this realization.

Other studies on plants differing in ploidy level include: the observation that autotetraploid tomatoes (Lycopersicon esculentum) are able to conserve water and amass dry weight under saline conditions better than their diploid progenital stock (Tal and Gardi, 1976); the fact

that triploid Thalictrum alpinum is more vigorous with respect to photosynthetic capacity than diploid members of the same population collected from the Ogotoruk Creek area (Mooney and Johnson, 1965); whereas tetraploid and diploid Viola adunca show no differences with respect to either photosynthetic capacity, or certain aspects of their water relations under varying temperature and moisture regimes (Mauer et al., 1978).

Another paper which, although not primarily concerned with ploidy, had considerable influence on the development of this Viola project, was that of Döbereiner et al. (1972) which dealt with the activity of bacterial nitrogen-fixers in the rhizosphere of the tropical grass Paspalum notatum. In their work they observed that only a tetraploid cultivar was able to establish the necessary association, and not the diploid. Coupled with a reference to nitrogen-fixation in the genus Viola (Alexander, 1977), and others to nodulation of V. praemorsa (Farnsworth and Clawson, 1972; Farnsworth, 1979), it was decided to include in the project a cursory look into the possibility of nitrogen-fixation occurring, with the recognition that an ability to establish an association with nitrogen-fixing microorganisms could well serve to assist a pioneer species in recolonization of the barren glacial till.

Mauer's thesis work (1977), which constituted a comparative eco-physiological study of the chromosome races of Viola adunca and formed the basis for the paper mentioned

above, was performed in the Dept. of Botany here at the University of Alberta. For this reason it was decided to use the plant material already available, and to attempt a furthering of the physiological characterization of a species already partially known.

Mauer, through a rigorous monitoring, was able to describe fairly fully the behaviour of the species with regard to photosynthetic capacity and water relations. A summary of his findings is presented in Table 1. The consideration of productivity, dealt with by Mauer in terms of net assimilation, was kept open in this study as a possible area of difference between the races and marked for investigation at phenological stages other than the mature plant.

As is mentioned above, the initial approach to expanding the comparative characterization of the chromosome races of Viola adunca, was to broach the specific question of cold hardiness in an attempt to test the Hagerup-based hypothesis that ploidy implied a greater tolerance of environmental extremes. After a period of observation of some of the behaviour of the two ploidy level populations however, it was considered most profitable to modify this approach, and to investigate the two major, and less rigidly defined categories of:

a. Cold adaptedness, and

b. Productivity,

both of which were used as frames of reference for

Table 1. A summary of the results obtained by Mauer (1977) in his investigation of the comparative eco-physiology of the chromosome races of Viola adunca. None of the results showed any significant difference between ploidy levels.

	Polyploid (3N & 4N)	Diploid (2N)
Maximum net assimilation of carbon dioxide at 20C and 500 uEinsteins.$m^{-2}.s^{-1}$ ($mg.(g\ dry\ wt.)^{-1}.h^{-1}$)	26	23
Dark respiration of carbon dioxide at 20C ($mg.(g\ dry\ wt.)^{-1}.h^{-1}$)	2.2	2.0
Mean max. water potential for both ploidy levels (bars).	-7.9	
Mean min. leaf resistance for both ploidy levels. ($s.cm^{-1}$)	3.6	
Water potential stress threshold for net assimilation for both ploidy levels (bars).	-14	

experimental research into all stages of the life-cycle (see Fig. 1). This allowed the formulation of a working hypothesis very similar to the general proposition of Johnson et al. (1965), in which they suggest two possible reasons for the observed change in the frequency of polyploidy with latitude. These possibilities, which should not be considered as mutually exclusive, are:

a. that polyploidy allows the development of a greater tolerance to environmental extremes; and

b. that polyploids are better able, through some intrinsic quality related to ploidy, to recolonize areas disturbed by the Pleistocene glaciation.

****

The general scheme thus established for what might be considered a comparative autecological study of the chromosome races of Viola adunca, can be described synoptically as follows:

1. Cold adaptedness of:
   a. Leaf tissue from mature plants;
   b. Seeds;
   c. Seedlings;
   d. Whole mature plants.
2. Productivity through:
   a. Seed production;

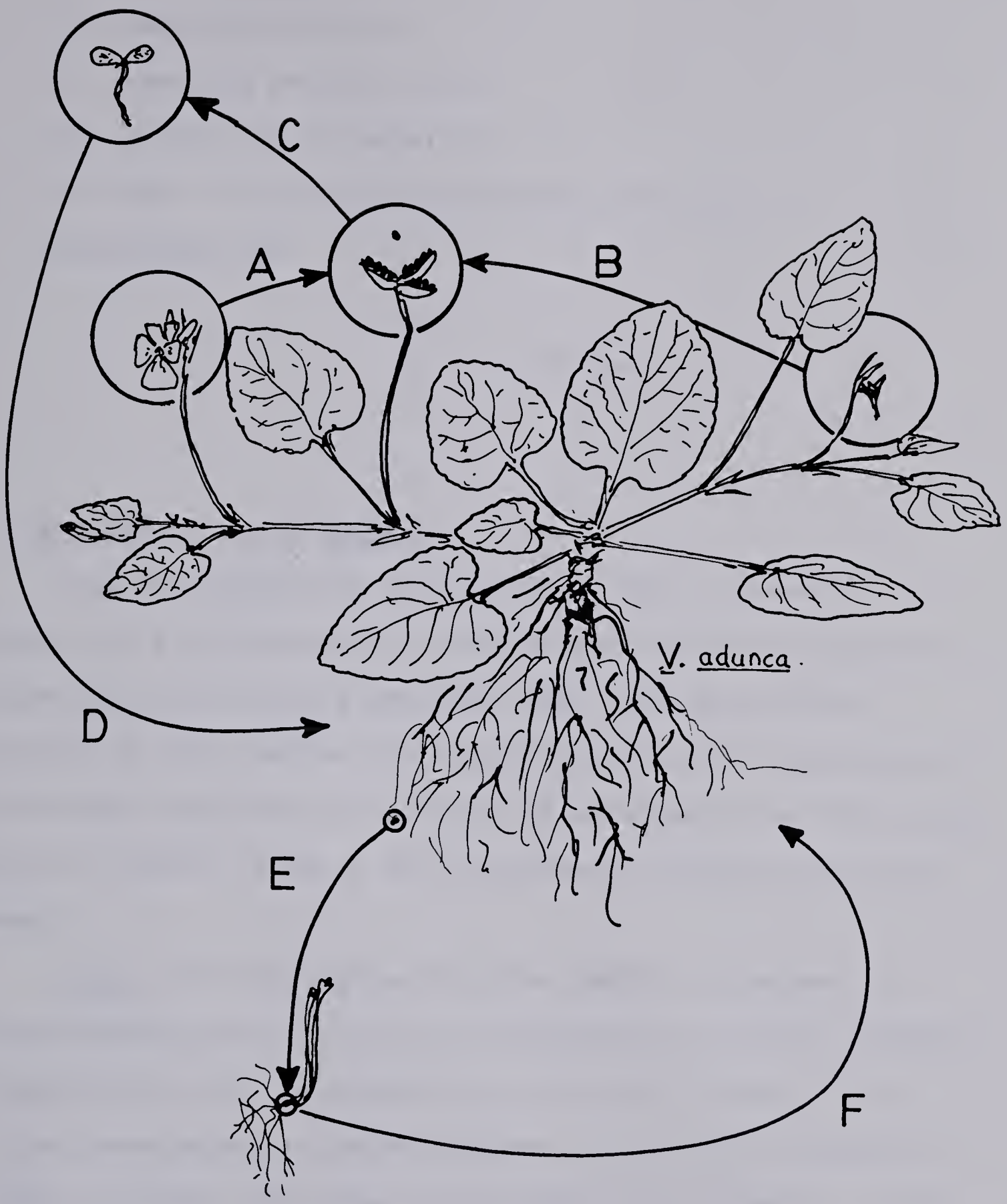

Fig. 1. The propagational strategies of Viola adunca. These are: seed production via (A) chasmogamous, and (B) the obligately selfing cleistogamous flowers. Given successful germination (C) and seedling survival (D), this appears to be the major channel for the regeneration of mature plants. Vegetative reproduction from overwintered root tissue is observed in the spring and early summer (E and F).

b. Seed germination;

c. Seedling productivity;

d. Vegetative propagation.

3. Nitrogen fixation by associated rhizospheric microorganisms.

****

A profile of Viola adunca.

The development of this project into a somewhat descriptive autecological study necessitated (and partially arose out of) a fairly detailed look into the natural history of the species. As a background to the experimental work described below, a profile is provided here which might help to explain some of the experimental design decisions made.

Viola, the type genus for the family Violaceae, is distributed globally, and is represented by species ranging from the familiar woodland herbs, to small shrubs, the latter considered to be evolutionarily relict (Valentine, 1962). Although the genus is systematically complex, with much hybridization to blur the boundaries between species, Viola adunca has been assigned a distribution primarily North American, without any specific links with European violets, as was originally supposed (McPherson and Packer, 1974).

Viola adunca is a perennial herb, represented by two chromosome races over its range (2N = 20, and 4N = 40), and is found in a wide diversity of habitats. Noticeably different environmental parameters include: soil moisture; soil organic-matter content; soil texture; shadedness of the site; and associated species. (e.g. From the arid grass-covered hills overlooking the Red Deer River near Drumheller, to the shaded leaf litter beneath an aspen stand in the Cypress Hills, to the open, sandy disturbed sites in the jackpine-lichen woodland of northeastern Alberta).

In their taxonomic contribution, McPherson and Packer described the distribution, showing the respective domains of the chromosome races (see Fig. 2), as well as characterizing a number of morphological differences, on the strength of which Löve and Löve (1975) proposed full species status for the tetraploid. These differences are in the size of both guard cells and pollen grains (in both cases the 4N-size is significantly larger than that of the 2N), and the morphology of the set of protuberances on the rounded head of the style, a feature typical of the genus. Although both races show great variability in the size and frequency of these stubby hair-like appendages, the 2N extreme is one of obviously greater density and length than the 4N. The function of these appendages is generally considered to be that of guiding insect visitors past the style, over the stigmatic cavity, and so to the nectary which is housed in the spur (see Fig. 3). This is the picture presented by

Fig. 2. Distribution of the chromosome races of Viola adunca in North America (●=diploid and o=tetraploid), showing the maximum extent of the Wisconsin glaciation (——-——-——). (After McPherson, 1972; McPherson and Packer, 1974).

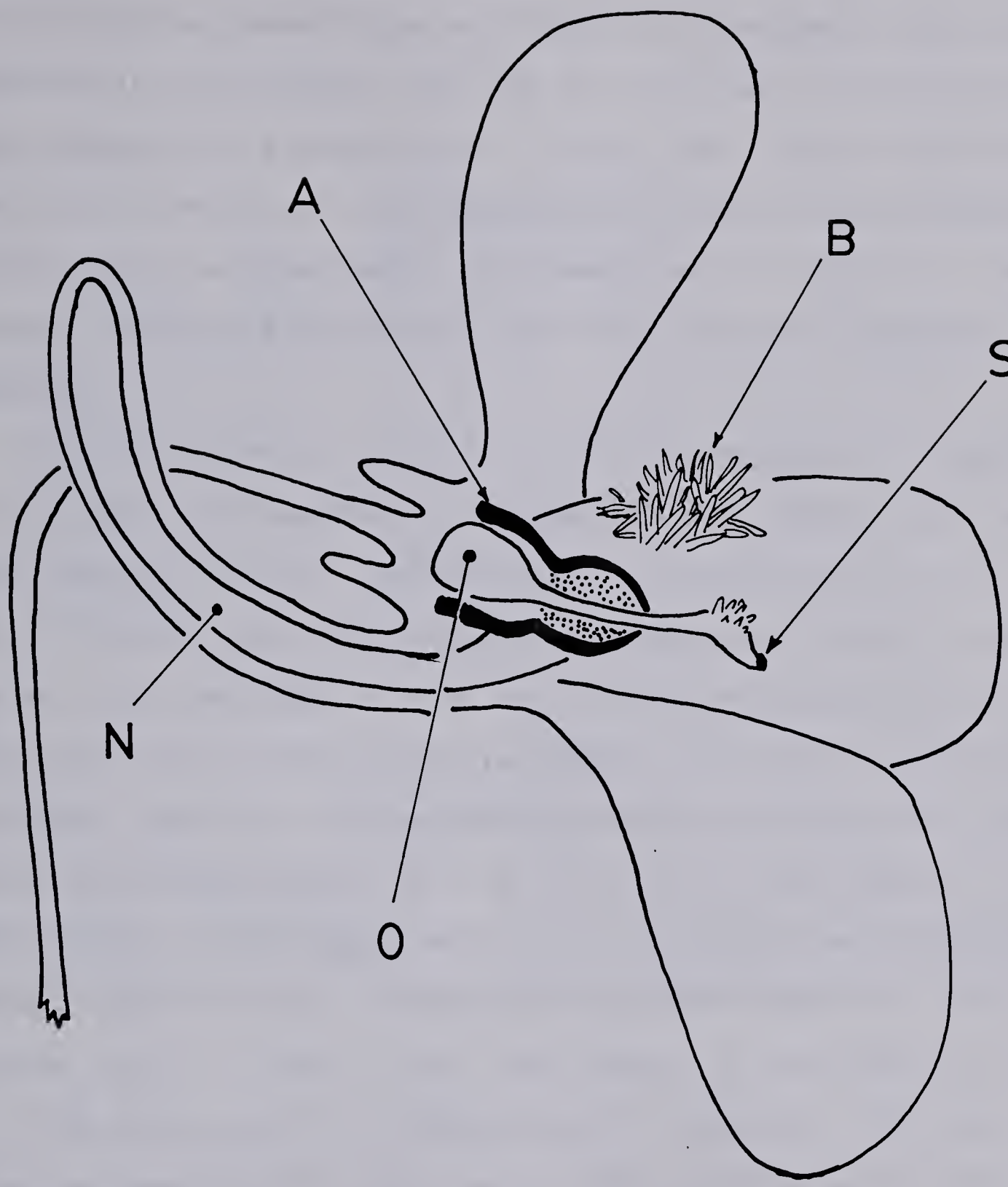

Fig. 3. The floral characteristics of zygomorphic Viola flowers showing: anthers (A); lateral petal "beards" (B); the stigmatic cavity (S); the ovary (O); and the nectary within the characteristic spur (N). (After Beattie, 1974).

Beattie (1974), who has done much of the work on the pollination and seed dispersal habits in the genus, and has observed in his studies that 89% of the insect visitors to Viola adunca are sternotribic[2] (ibid). The "beard" hairs on the lateral petals of the zygomorphic flowers are claimed by Beattie, in the same paper, to present secure grips for the insects reaching the nectary from this otherwise awkward position.

As will be made apparent in the presentation of results below, open (chasmogamous) flowers were not found to be the major source of seed. Instead seed is produced mainly via the obligately self-pollinating cleistogamous flowers, which are entirely enclosed within their arrowhead shaped calyx until swelling of the gynoecium begins. The mature capsules, which are identical to the chasmogamously produced ones, are three valved and can contain up to 30 dark, hard-coated endospermous seeds approximately 1mm in length and ovoid in shape. (Seeds of both chromosome races were found in this current study to have a cold requirement for germination.)

The seed capsule is pendant while ripening, but just prior to opening will adopt an upright position such that when the valves separate, the seeds are arranged perched atop three radial arms before being forcibly discharged by the pinching action of the valve as it dries (see Fig. 4).

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[2] Sternotriby describes the situation where insects, mainly the solitary bees, alight on the top of the flower and attain the nectary by reaching right over and inserting their probosces from an inverted position, thus making contact with the stigmatic area with the ventral sides of their bodies.

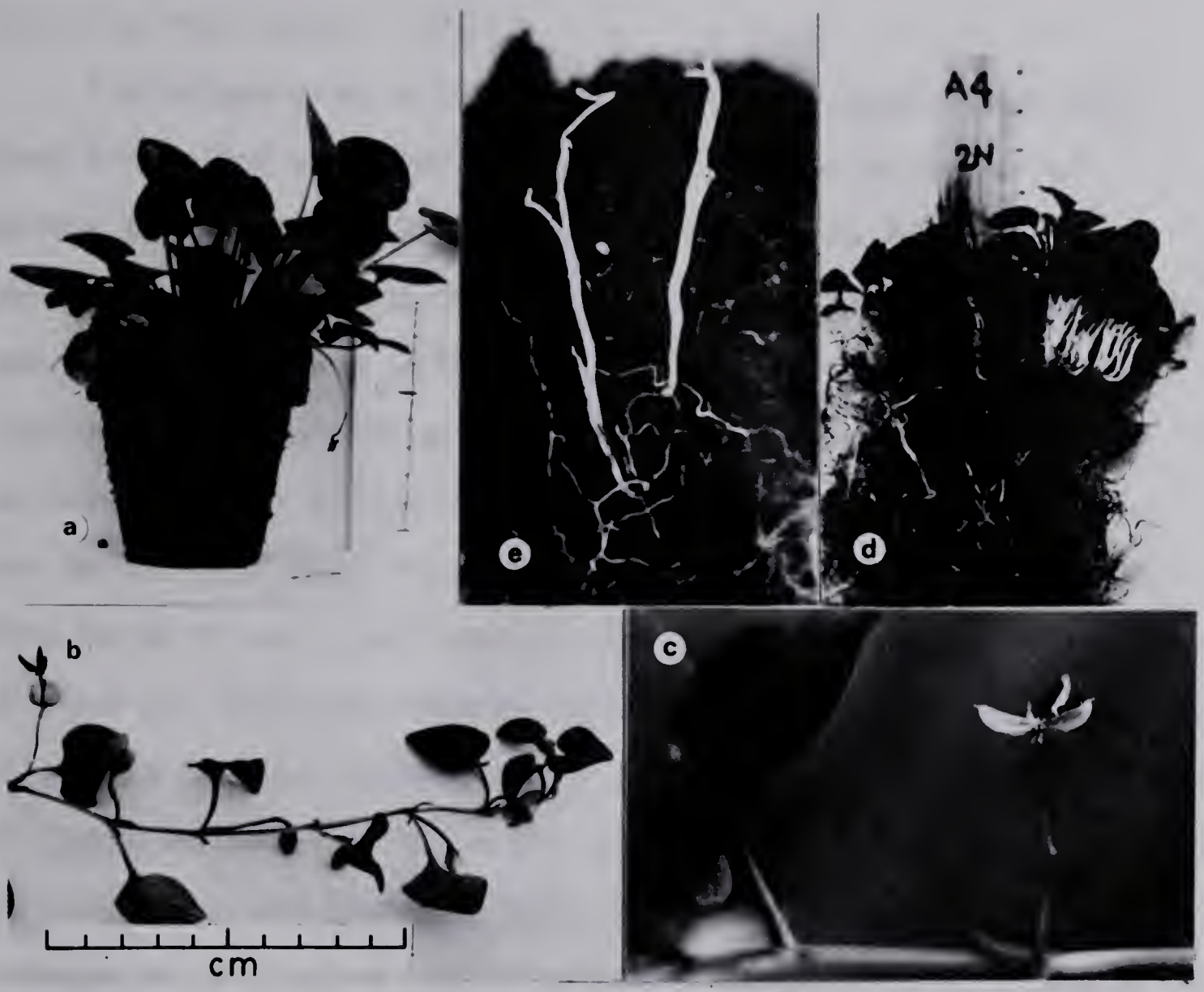

Fig. 4. Viola adunca. (a) A typical 2N plant displaying a habit more compact than the 4N, which more commonly produced trailing shoots (as is seen in b) under greenhouse conditions. (c) Seed capsules of Viola adunca, showing the adoption of an upright position just before dehiscence. (d) and (e) Plants subjected to winter out of doors tended to regenerate vegetatively from root tissue of the previous year's growth, all above-ground tissue having died back.

Seeds thus discharged can travel about three or four metres, and each is equipped with a small caruncular elaiosome (sic) which is possibly connected with further dispersal by ants (Beattie and Lyons, 1975).

Cleistogamy as a flowering habit has been known for some time, and was described in detail for a number of genera by Darwin (1892)[3]. In the same essay he lists 46 genera of dicots, and 10 of monocots displaying cleistogamy, and deals in detail with five species of Viola. Based on typically meticulous observations, he regards cleistogamy partially as an arresting of the developmental processes, but points out that in many cases specific adaptations to this mode of self-pollination can be discerned. (An instance of this is "the hook-shaped pistil in Viola ... by which the stigma is brought close to the fertile anthers". The species of Viola he deals with in his survey are: canina, odorata, hirta, nana, and Roxburghiana, the last two being species indigenous to India). Most commonly associated with weedy annuals but often with other pioneering species of composites and grasses (Jain, 1976), self-pollination is a habit which, when manifest in its extreme form of cleistogamy, allows a higher turnover of propagules at a reduced cost (Schemske,1978). The relevance of these observations to Viola adunca forms part of the basis of this study, and the implications are considered below in the

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[3]Darwin in turn gives most of the credit for the recognition and documentation of cleistogamy to the German workers, Hugo von Mohl and Dr. Kuhn.

Discussion.

Plants used in this study were collected in the Cypress Hills of southeastern Alberta, and had been under greenhouse cultivation (at the U. of A., in the Botany greenhouses) from September 1975 until the commencement of this study in September 1977. Between the two studies, they were propagated accidentally by seed (presumably mostly cleistogamous), and intentionally by cuttings. The gross morphology of these plants cultivated under identical conditions, showed one striking difference between the chromosome races. Tetraploid plants consistently adopted a somewhat trailing habit, producing shoots up to 30cm in length, whereas the diploids were mostly more cushion-like in appearance, with much shorter cauline shoots (see Fig. 4 on page 17), although trailing shoots were sometimes observed in the latter race. (Voucher specimens of each race of the greenhouse material have been deposited in the herbarium at the University of Alberta.) Neither race rooted readily from shoot tissue which did not include at least some of the crown tissue, and therefore the trailing habit of the 4N should not be considered as a candidate for stoloniferous propagation.

A certain amount of propagation seems to occur very locally in the spring by means of shoots propagated from the root system established in previous years (see Fig. 4). Under severe winter conditions, the dense crown, from which the monopodial, alternatively leaved shoots arise, will die

back, and growth be resumed by the newly formed plantlets. (See Discussion for the details of this phenomenon).

II. Methods and materials.

A. Cold adaptedness.

1. Leaf tissue.

Cold hardiness of leaf tissue was determined for plants under various growing regimes. (for details see Table 2 on page 46). During the course of the investigation, two methods were employed for the determination of lethal stress. The first, which was later abandoned for a simpler and more practical method, was similar to that described by Steponkus and Lanphear (1967). Here tissue samples in the form of leaf discs were excised from cold stressed shoots, infiltrated under vacuum in a triphenyl-tetrazolium-chloride (TTC) buffer solution, and incubated at 30C for 15h. The reduced TTC, a red formazan, was then extracted with hot 95% ethanol, and the extract made up to 5ml. The absorbancies of these solutions were then measured on a Beckman spectrophotometer (DB-G) at 530 nm, and TTC reduction expressed as the ratio of the absorbancy at the stress temperature to that of an unstressed control. The control plus each stress temperature was represented by 5 leaf dics from 5 different plants, the stressed absorbancy always being compared to the control absorbancy for the same plant. This procedure was paralleled for each ploidy level, the choice of leaves for stressing being standardized as "mature and non-senescing". Leaves were picked from the test plants, wrapped in aluminium foil to prevent desiccation, and then

subjected to the stressing cold by being placed in an M3 controlled enviroment cabinet (CEC) with good cooling capabilities. (For details of the controlled environment facilities used, see Appendix II). For 0C stress, the temperature was held at freezing for 2h, after which time samples were removed, and temperature control turned to the remote controller. This was equipped with a cam cut to give a decrease in temperature of 1C per hour. Stress samples sets were then removed at approximately 5C intervals, the lowest temperature reached being -21 C. Also inside the CEC were open thermos flasks, one for each stress temperature, into which samples were placed, and the lid closed before removal to the cold room at 3C ± 2C where the samples were left to thaw for 12h. Sample temperatures were monitored using fine wire thermocouples (.125mm; Omega Engineering, Stanford, Conn.) in conjunction with a Fluke digital thermometer (model 2100A). It was determined that sample temperatures coincided closely with air temperatures inside the CEC, and that warming inside the thermos-flask occured at between 3C and 5C per hour. This was the method used for one experiment, an estimate of cold hardiness being obtained for unhardened plants at the start, and then again after 4 weeks of short days (8h), and progressively cooling temperatures in an M7 CEC. (See Results, Fig. 10 on page 49, for the details of light and temperature). The usual way in which this method is calibrated, is for TTC reduction to be compared to the rooting ability and survival of cuttings.

Viola adunca however, proved to be an erratic rooter from cuttings, which made calibration of the method by this means difficult. It was therefore decided to calibrate the TTC method against the survival of leaf discs floating on full-strength Hoagland's solution (Hewitt, 1966). This visual rating of stress damage proved successful and conservative enough with respect to the amount of tissue required, to be adopted as the primary test (see below for details). Calibration points obtained by this means, and a few more from whole potted plants (see Fig. 5) did, however, indicate a correspondence to the calibrations of Steponkus and Lanphear(1967) and Wilkinson (1977), both of which gave 50% absorbance as the critical ratio. Results for the two hardiness values obtained in this manner, and the method of interpolation, are shown in Figs. 6 and 7. For all subsequent cold hardiness tests on leaf tissue, the disc-on-Hoagland's means of evaluation was adopted. This type of browning test is regarded by Stergios and Howell in their review of cold hardiness tests (1973), as highly accurate. The browning of the tissue is normally attributed to the action of polyphenoloxidases whose substrates are released from the vacuole on disruption by cold stress of the tonoplast (Mayer and Harel, 1979). In addition, it was decided to change to a more precisely controllable method of stressing, by employing two available temperature baths (Polyscience). For this method, leaf discs 6mm in diameter were excised from unstressed test plants by means of a

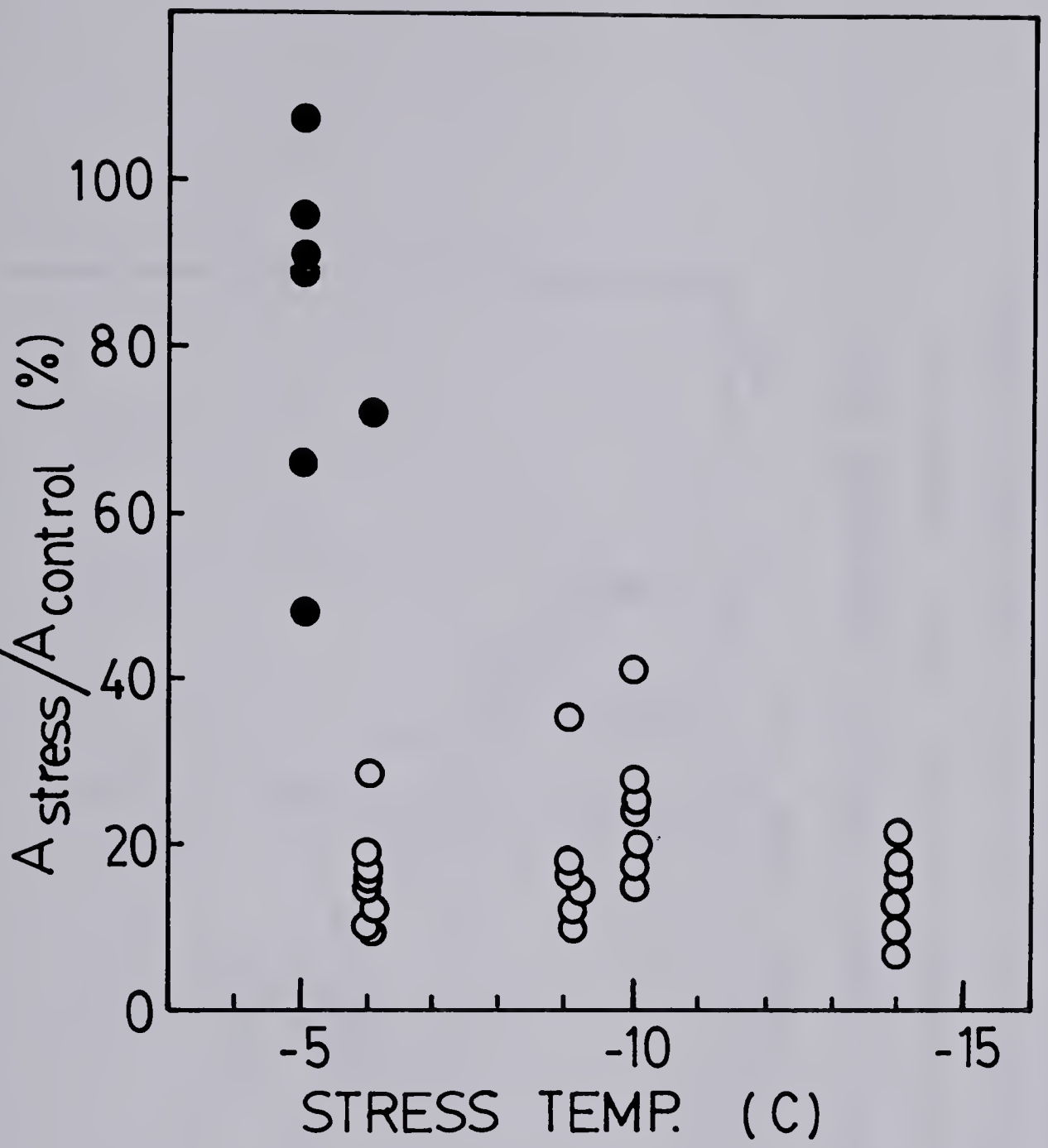

Fig. 5. Calibration of the TTC method for the determination of cold hardiness. Survival (●) and non-survival (o) of sample leaf discs for given degrees of cold stress are plotted against the absorbance of the reduced TTC at 530nm, relative to unstressed controls.

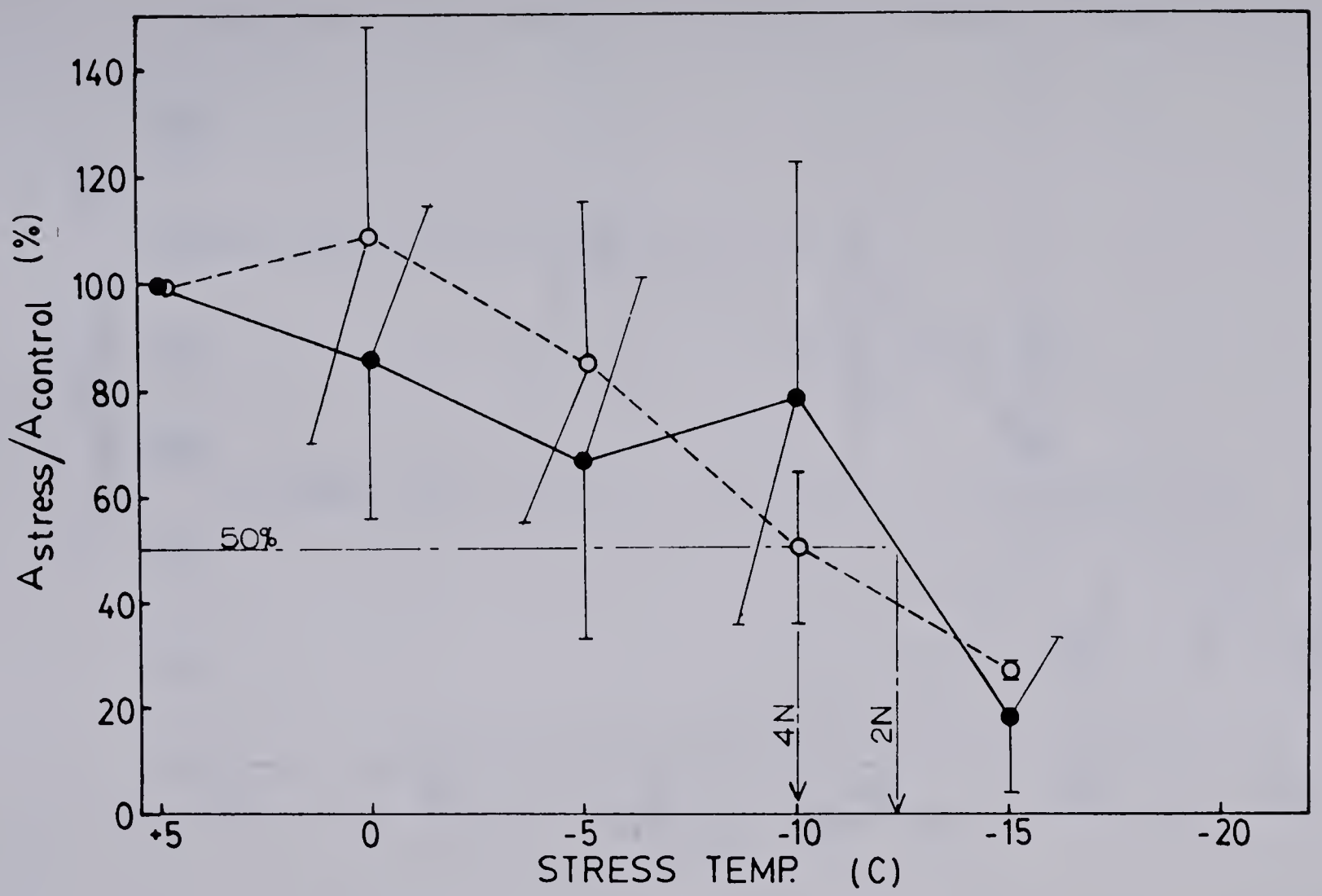

Fig. 6. Absorbance ratios at 530nm for unhardened leaf tissue, showing the nominal critical stress temperature which gives 50% absorbance relative to unstressed controls (●=diploid and o=tetraploid). Vertical bars indicate the 95% confidence interval.

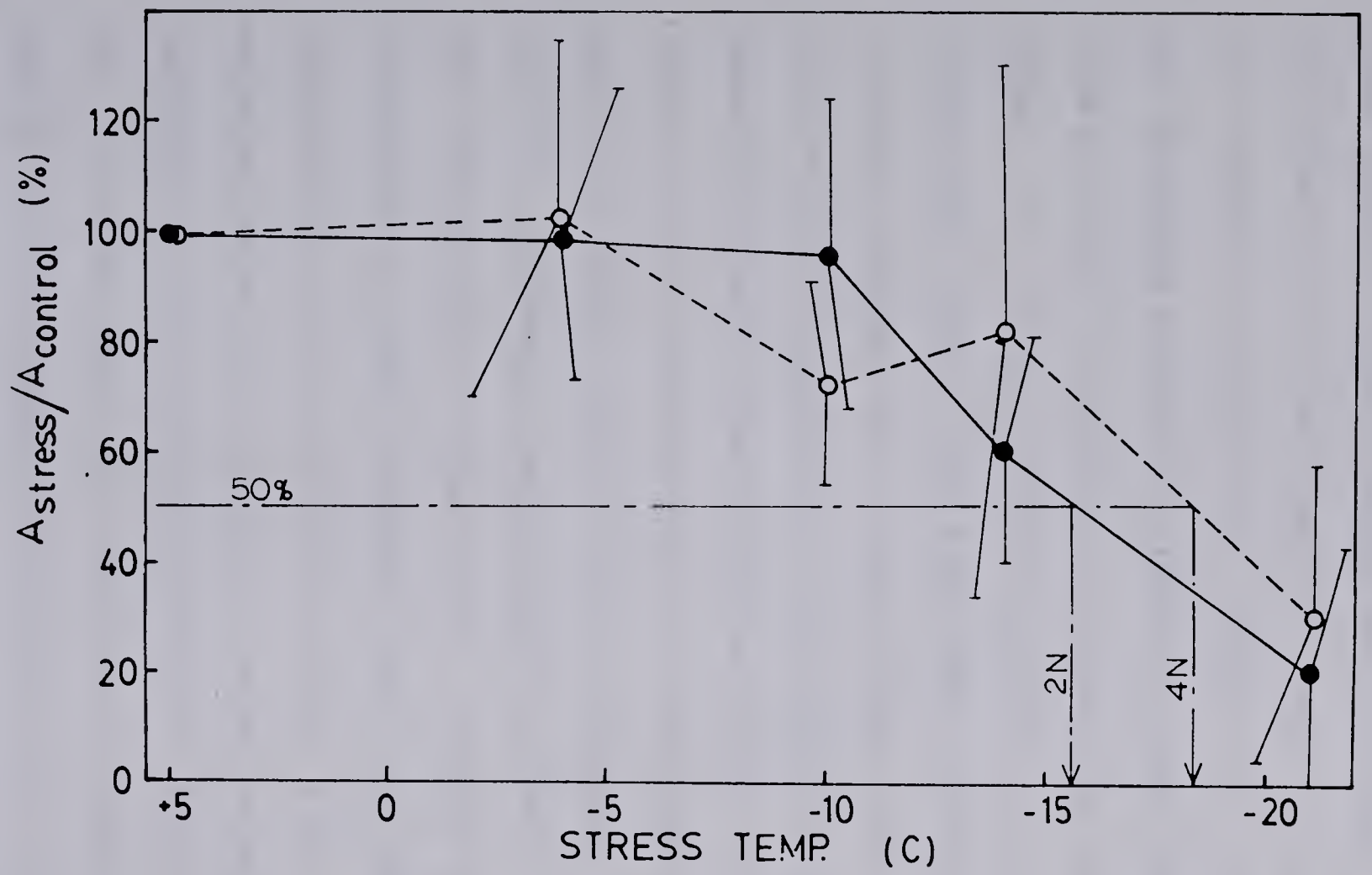

Fig. 7. Absorbance ratios at 530nm for hardened leaf tissue, showing the nominal critical stress temperature which gives 50% absorbance relative to unstressed controls (●=diploid and o=tetraploid). Vertical bars indicate the 95% confidence interval.

sharpened cork-borer. The sampling pattern was the same as that described above for the TTC test, only where possible, juvenile leaves not yet fully expanded were picked *in toto*, and formed a parallel sample set. Discs (plus juvenile counterparts when present) were then wrapped individually in aluminium foil, and all such samples for any one treatment wrapped in a second foil envelope. These were then wrappped around cylindrical brass heat sinks 3cm in diameter and 4.5 cm high, secured with masking tape, and placed in a lidless aluminium cannister 5cm in diameter and 8.5 cm high. One or two fine wire thermocouples were placed in dummy packages as for leaf discs, for the precise monitoring of temperature. Once equilibrated, this temperature was found to follow very closely (± 0.5C) the temperature of the methanol in the temperature bath. The cannisters were placed into the methanol such that they were partially submerged (approximately 6cm), the temperature bath having been pre-set to 0C. Samples took approximately 30 minutes to reach freezing, and were kept at this temperature for a further 2h. After this, samples representative of a 0C stress were removed and the temperature bath set for cooling at approximately 1C per hour. This decrease was achieved by mechanically coupling an appropriately geared synchronous motor (Cramer) to the thermoregulator control. Stress samples were removed at temperature intervals (3C to 5C) over a range within which

the critical stress temperature was thought to lie. When a sample set reached its nominal stress temperature, the cannister was transferred quickly to the second methanol cold bath pre-set to that stress temperature. The temperature of this second bath was then raised at a rate of 3C to 5C per hour in the same fashion, using a differently geared motor. On reaching a temperature between 0C and +3C, the cannister was moved to the 3C cold-room mentioned above, where a 12h thaw period was once again allowed. To determine injury, each thawed tissue sample was placed in a 5cm petri dish containing full strengh Hoaglands' solution and visual assessment made after 10 days in a germination cabinet set for a 16h light period at 20C, followed by a dark period at 10C. The 8 fluorescent lamps provided approximately 75microEinsteins.$m^{-2}.s^{-1}$ of photosynthetically active radiation (400-700nm; PhAR). (see Appendix II). The set-up might be descibed as two matrices, one for each ploidy level, the rows being stress temperatures, and the columns being test plants. (The first row, as for the TTC test, was an unstressed control, subjected to each step of the procedure except stressing). Tissue necrosis was usually uniform across a disc, but where patchy, the area of necrosis was estimated. In this way a "percentage necrosis" for each row was easily determined, and an estimate of the stress temperature giving 50% necrosis interpolated. This estimate was taken as the "50%-Lethal Dose" (LD-50), and regarded as a measure of the cold hardiness of the leaf

tissue.

2. Water relations associated with hardening.

For one experiment during which plants were hardened off in an M7 CEC with decreasing temperatures over a four-and-a-half week period (see Fig. 10 on page 49) leaf samples were taken at 7 to 10 day intervals, and their water potentials determined. This was done by the ballistic method of Spanner (1951) in stainless steel chamber-psychometers (Mayo 1974), which had been calibrated against filter paper discs soaked in sodium chloride solutions of known water potential (Slavik, 1974). For each data point, a sample leaf disc 5mm in diameter was taken from one mature non-senescing leaf from each of five plants. This was done for each ploidy level. The ten loaded psychrometers were then placed in polythene bags and allowed to equilibrate immersed in a stirred, but unheated water bath. Output potentials were measured after 6h on a Fluke microvoltmeter (model 845AB) linked to a custom built switching- and cooling-circuit (see Fig 8). A cooling current of 7mA was passed through the bead for 10s in order to effect the condensation required for the establishment of measurable output. In order to determine the component of water potential attributable to matric and osmotic forces, chambers were removed from the psychrometer after the initial total water potential readings, wrapped quickly and tightly in aluminium foil, and immersed in

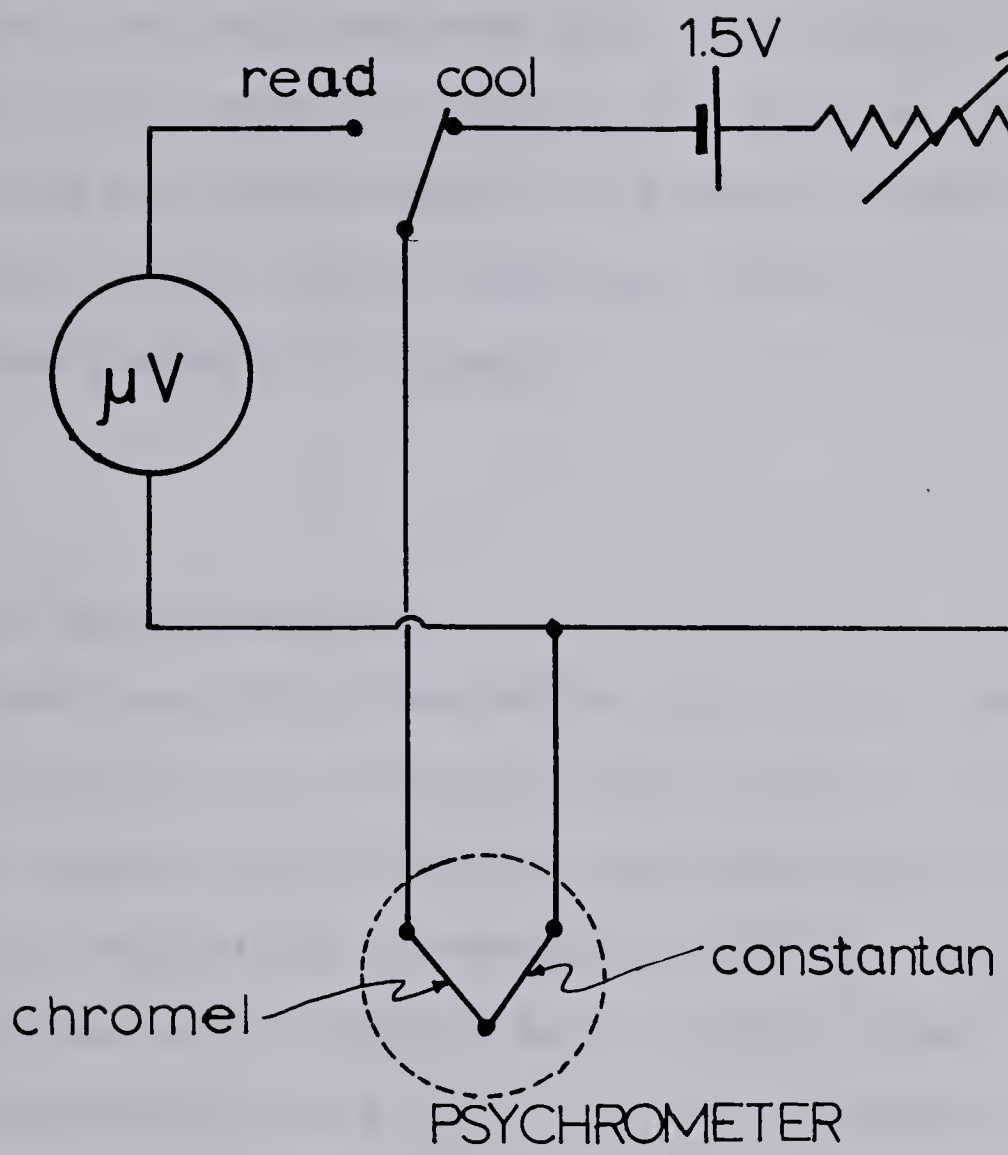

Fig. 8. Schematic diagram for the cooling and reading circuits used with the tissue psychrometers in the determination of leaf water potential. (After Spanner, 1951).

liquid nitrogen (-196C) for 10 minutes. After removal they were allowed approx. 15 minutes to equilibrate to room temperature and then unwrapped and returned to their appropriate psychrometers. The measuring procedure was then repeated for these membrane disrupted samples. After this, chambers were emptied, and all psychrometer parts washed in distilled water and dried in a stream of warm air supplied by a hair dryer (440W). They were stored in a dust free enviroment when not in use.

3. Seed cold hardiness.

Seeds were pre-treated in the fashion described below under Productivity - Germination. In the initial pilot study, (moist) stratification was limited to the 3 weeks in the cool trop-arctic greenhouse, and the seed-coat of each seed nicked with a razor blade at the chalazal end such that the the endosperm was just visible (treatment II in Table 10 on page 68). In each of several petri dishes, ten such seeds were placed on a double layer of no.1 filter paper (Whatman) which had been soaked in a suspension of Captan (1g per 1) to suppress fungal infection. Excess moisture was drained off so that the filter paper was no more than damp. A complete sample set, one for each ploidy level, consisted of 3 petri dishes per temperature treatment, and 6 unstressed controls. Stressing was accomplished in the M3 CEC described above, by simply presetting the temperature to

the stress point, and placing the sample set inside for 6h. Immediately after this, the petri dishes were placed in the germination cabinet where they were left for the following 2 weeks, being moistened with distilled water when necessary. At the end of the 2 week period, germination success was determined.

A second, more comprehensive test was carried out with seeds collected from the CEC productivity run (see Productivity below), as opposed to the first test, where seeds from outdoor plants were used. Stratification was augmented by a 6 week period in the +3C cold room (treatment III in Table 10 on page 68), and seed-coats were not artificially broken. In addition, stressing was achieved by equilibrating the samples at 0C for 2h and then switching to the remote controller which was equipped with a cam designed to lower the chamber temperature at 3C per hour. On reaching the nominal temperature, the cam was reversed, and the CEC temperature raised at the same rate to a temperature between 0C and 3C. From here samples were moved to the 3C cold room and allowed to thaw for 12h before being placed in the germination cabinet. The exceptions to this procedure were the 0C and -5C sample sets, both of which were given 5h at 0C in order to compensate for the duration of stress. A complete sample set, one for each ploidy level, was a 7x7 matrix, columns being random sets of 10 seeds per element, and rows representative of 6 stress temperatures and one control. Germination success was again assessed after 14

days.

A third test was conducted on seeds subjected to an even longer and colder stratification, which consisted of the 9 week period described above, plus a storage period of 6 weeks at -3C (treatment IV in Table 10).

4. Seedling cold hardiness.

a). Unhardened seedlings (14 days old).

Seeds from an intermediate scarification treatment (treatment III above), were germinated under equitable conditions in the germination cabinet. After 2 weeks, germinants were placed in 2 common beakers (one for each ploidy level), containing Hoagland's solution, and then picked out one by one at random, and placed in 9cm diameter petri dishes, 5 seedlings per dish, on fresh filter paper soaked in Captan suspension. For each stress temperature, samples of 3 dishes were taken from each ploidy level set, and subjected to cold stress in the M3 CEC. The temperature was lowered at 3C per hour to the nominal stress temperature, and then raised again at the same rate. Survival was assessed after a further 14 days in the germination cabinet.

b). Hardened seedlings (7-8 weeks old).

As in (a) above, seedlings were germinated, and seedlings rearranged randomly in petri dishes after 2 weeks, this time 9 seedlings to a dish. These were then returned to the

germination cabinet where they were kept moist with Hoagland's solution. After one week the seedlings were transferred to fresh Captan soaked filter paper, and returned to the germination cabinet for another week. The dishes of 4 week old seedlings were then transferred to the M7 CEC set up for the following regime: a 16h light period (250 microEinsteins.$m^{-2}.s^{-1}$ PhAR ± 10%) at 12C ± 1C, followed by a dark period of 8h at 7C ± 1C. After 10 days the light and dark temperatures were reduced to 7C and 3C respectively (± 1C). Between the twelfth and seventeenth day of this cooler regime, dishes were removed and stressed in the same fashion as the second seed test above. Sample sizes were either 2 or 3 petri dishes per ploidy level for each stress temperature. Survival was assessed after 14 days. (There was some problem with algal growth - species not determined - once Hoagland's solution was introduced, but seedling growth did not appear to be affected). Seedlings were inspected again after a further 14 days before being discarded.

Because of the unavailability of CEC facilities and the need to share space with other users, it was not possible to shorten the photoperiod of this hardening regime. But this situation, it was felt, provided the opportunity to investigate the degree to which seedlings could be temperature hardened during the equivalent of the early part of a growing season, when unseasonal frosts might occur in nature.

5. Whole plant survival after cold stressing.

Although the leaf tissue hardiness tests were able to reveal some aspects of the hardening ability of mature plants, they left untouched the important question of the hardiness of the perennating parts of the plant viz. the crown and the root system in the intact plant. For this reason, a set of experimental plants were propagated by cuttings from plants of known chromosome number, that having been determined by the root tip squash method described in Appendix I. Cuttings were made in November 1978, treated with a rooting hormone (Rootone No.2; Amchem Products, Ambler, Pa.), and set to root in flats of sand. These flats were placed in a propagation greenhouse with supplementary lighting, which consisted of a mixture of 400W Lucalox and 400W Multivapor (General Electric) at approximately 1.3m above the bench, and fertilized weekly with Hoagland's solution (Hewitt, 1966). After 7 weeks the flats were placed in the cool trop-arctic greenhouse (254 degree hours per day) under natural light conditions for 4 weeks, in order to vernalize the surviving rooted cuttings. Plants thrived on their return to the propagation room, and cuttings were potted into 6cm diameter clay pots in a standard potting mix (2:2:1), after a further 7 weeks. On March 16 they were placed in a standard greenhouse under natural lighting and given an application of NPK fertilizer (20:20:20). Between June 2 and 9, sample sets of 3 plants for each ploidy level were chosen at random with the aid of a random numbers table

(Fisher and Yates, 1948), and subjected to the same stress and thaw procedure as were the seeds and seedlings described above. A soil thermocouple indicated that the soil temperature was the same as that of the air in the CEC at the termination of the stress period. Instead of being placed in the germination cabinet, plants were returned to the greenhouse and given their regular waterings as before. Assessment of damage was made after 14 days, and then again after 7 weeks. A similar procedure was adopted for "hardened" plants, these being plants of the same batch treated with short days and cold temperatures for a period prior to testing. The hardening regime consisted of 4 weeks of short days (8h) at 15C with dark periods at 5C, the latter 2 weeks having 2 hours of -2C frost in the mid-dark period. Light throughout was 275microEinstiens.$m^{-2}.s^{-1}$ (PhAR) and relative humidity, though not controlled, was at approximately 50%.

6. Outdoor plants.

Mature plants in mid-growing season, whose chromosome number had been determined directly by counting, were moved from a standard greenhouse to a south-facing alcove on the roof of the Biological Sciences building (at the east end of the sixth floor greenhouses) in late July, 1978. Here, their 7cm diameter pots were embedded in the peat contained in large frames, where they received regular watering. The

diploid and tetraploid sets of plants consisted of 10 and 11 plants respectively. These plants were left here for the winter of 1978/1979, accompanied by a maximum/minimum thermometer (Taylor) lying close to horizontal on the surface of the peat. The lowest winter temperature recorded by the minimum rider was -32C. New growth was observed in the unusually late spring of 1979, a careful watch being kept for germinating seedlings. These were ruthlessly weeded out when occurring inside the pots. On July 11 all plants were depotted in order to inspect the subterranean morphology.

B. Productivity.

1. Seed production by cleistogamy.

From the outdoor plants described above, seed capsules were collected at two or three day intervals in the late summer and early fall. The three-valved capsules were considered ready for picking once they had assumed an erect position, before which, like the flower, they pointed downwards (See Fig. 4 on page 17). Plants of each ploidy level population were sampled collectively, and capsules placed in small paper envelopes where they were allowed to dehisce(approximately 24h). During the following weeks, the envelopes were opened and seeds and capsules counted. A second and statistically more rigorous measurement of seed productivity was made on another set of plants, which had been propagated by cuttings from stock of known ploidy

level. These plants in mid- to late-growing season, were moved from the greenhouse and placed in the CEC under long day conditions. (For details see Fig. 15 on page 62).

An explanation of the complicated changes in the regime is warranted. After approximately 2 weeks, the leaves started to bleach, so one third of the lights were switched off, the assumption being that 16h at 475 microEinsteins.$m^{-2}.s^{-1}$ was an overdose of light energy. After six weeks, although plants were doing satisfactorily, there was a tendency for the soil to dry out in spite of regular watering. This pointed to a problem with the humidification system in the chamber, which was unable to raise the dew point above 14C (approximately 65%rh), due perhaps to the dryness of building air during the winter months. For this reason a continuous 20C was adopted for three weeks for both dark and light periods, followed by lower temperatures to lead plants into dormancy. Seed capsules were picked as before, but this time the number harvested from each plants was recorded, thus enabling an estimate to be made for the number of seeds produced by each plant.

2. Germinability.

Seeds from the above-mentioned studies, designated as "outdoor" and "CEC" seeds, were stored under ambient building conditions in the dark for a minimum of 10 weeks

(during the winter), before commencement of germination tests. Preliminary tests revealed that a cold treatment was essential for germination, as well as other factors which appeared to influence success. By a series of trial and error experiments, the following standard pretreatment was arrived at:

a. tumbling in a rotating drum lined with coarse sandpaper, with about 2cc of coarse sand, at 120 rpm for 12h;
b. 24h imbibition under vacuum in a .05% Triton-X solution;
c. 48h leaching in 200ml distilled water with continuous agitation; and
d. 3 weeks of stratification under natural winter light in the cool trop-arctic greenhouse.

This last step had seeds in petri dishes on 2 sheets of filter paper (Whatman No.1), soaked liberally with a Captan suspension (1g per 1 in distilled water) to inhibit pathogenic fungal growth. From here a number of routes were taken. Germination tests were carried out at this stage on seeds with intact and cut seed-coats, or they were subjected to a further 6 weeks in the 3C ± 2C cold room before being laid out for germination. For the results presented in Fig. 17, sample sets consisted of 5 petri dishes per ploidy level, each containing 15 seeds. All other germination tests were conducted in conjunction with cold hardiness tests and are described above under cold adaptedness on page 31.

3. Seedling Productivity.

Successful 14 day old germinants of unstressed outdoor seeds were transplanted to compartmented seedling trays, into a mixture of sand and vermiculite (1:1), one per compartment, which measured 3.5 x 2.5 x 5.5cm deep. These were soaked in Hoaglands' solution and then placed in the propagation greenhouse on a heated bench approx. 1.5m beneath a row of 400Watt mercury lamps at 1m centres, which were switched on for 18h per day between 0200h and 2000h. Samples were harvested after 50 and 57 days, (in April 1979), washed clean of soil, and dried overnight in an oven at 70C. Roots and shoots were then separated and weighed separately on a Mettler H10 analytic balance, to yield information on total productivity, as well as root: shoot ratios. A second and similar experiment attempted to seek information about the relationship between productivity and temperature regime. For this, 21 day old germinants of the second, more comprehensive cold stress tests (see above), were placed in common pools, and picked at random for transplanting to seedling trays as before, but the medium this time was a 1:1 peat/vermiculate mixture with better water holding properties. Planted seedlings were sprayed with approximately 2ml each of methylmercury dicyandiamide solution (5ppm) about the roots to combat fungal infection. One tray (12 seedlings) of each ploidy level was placed in

each of the trop-arctic greenhouses[4], and harvested as before after 50 days. This experiment was conducted under natural lighting between late June and late August. The soil was kept moist by standing the trays in saucers of Hoagland's solution.

C. Nitrogen Fixation.

The preliminary investigation into the possible association of V. adunca with nitrogen-fixing soil microorganisms was conducted in two parts. The first was initiated in late August 1978, when a trip was made, and living plants were brought back from locations in Alberta and British Columbia (see Fig. 9). These plants were kept under standard greenhouse conditions in plastic pots in their native soil, and watered only with distilled water. Additional plants were obtained from Colorado, Montana and Sterco - Alberta, by people visiting these locations (see Preface for acknowledgements).

The method used to assay nitrogenase activity was the same as that used by Döbereiner et al.(1972), which utilizes the ability of nitrogenase to reduce acetylene to ethylene, thus allowing measurement of nitrogenase activity by gas chromatography (Hardy et al., 1973). In December, with the availabilty of a gas chromatograph (GC)[5], samples were taken

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[4] For details of the trop-arctic regimes, see Appendix II.
[5] This GC was of the portable type, built by Technical Services at U. of A. according to the design of T.M.Mallard, C.S.Mallard, H.S.Holfield, and T.A.LaRue at the Prairie Regional Laboratory of the National Resarch Council of Canada, in Saskatoon.

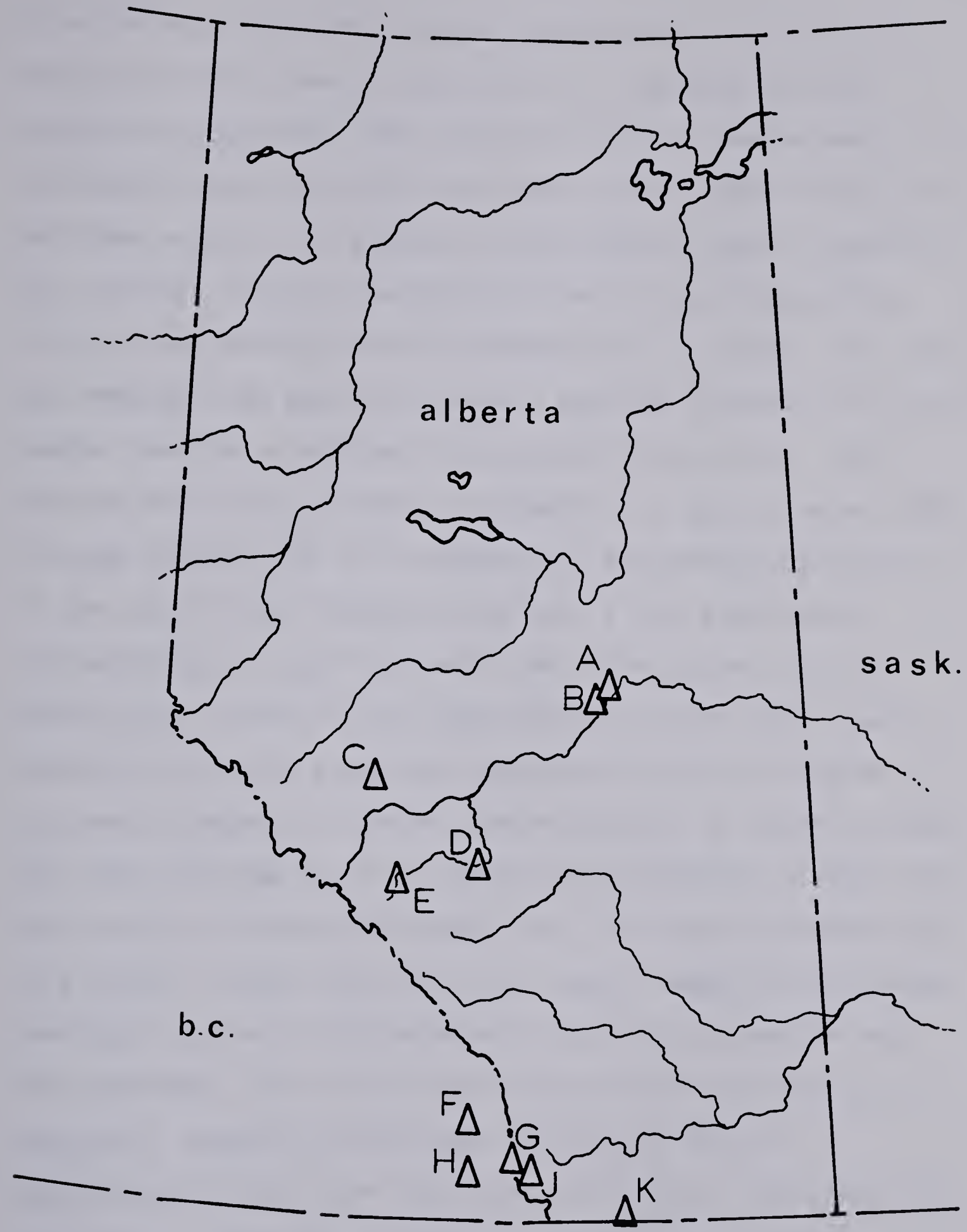

Fig. 9. Collection sites in Alberta and British Columbia of plants used in the assessment of rhizospheric nitrogen-fixation in Viola adunca. (See Tables 13 and 14).

from the roots of these plants, consisting of a clump of roots with the closely adhering soil. The samples were placed in individual 32ml screw-top vials. Samples were moistened liberally with sterilized, double distilled water and then sealed with snugly fitting rubber serum stoppers, the internal pressure being equalized by piercing with a fine syringe needle once the stopper was in place. Air (5ml) was removed from each vial with a syringe fitted with a fine needle, and 5ml acetylene introduced in its place. The samples were then allowed to incubate for approximately 48h at room temperature in the dark with continuous agitation. At the end of the incubation period, a 1ml sample was extracted by syringe from each, and introduced into the sample port of the GC, the peak height at the approximate retention time for ethylene recorded, and this compared to ethylene standards of known concentration. In order to check that the ethylene was not endogenously produced, rather than the product of acetylene reduction, vials were opened after this assay, flushed out with air, and allowed to stand open overnight before being resealed. The assay procedure was then repeated, only this time without the introduction of acetylene, samples being taken and run on the G.C. approximately 48h after resealing. This test indicated that significant endogenous ethylene production could be discounted. A further introduction of acetylene showed renewed (although supressed) ethylene production, thus confirming the result.

A second field trip was undertaken in late June 1979, when some of the Alberta and B.C. sites were revisited by car. Samples of root tissue and adhering soil, together with rootless soil control samples, were collected at each site, placed in vials, moistened, and sealed as before. These were then stored in test-tube racks above ice in an insulated chest, the air temperature inside fluctuating diurnally between approximately 2C and 15C, as indicated by a maximum/minimum thermometer. This arrangement was in effect until returning to the city 48h after the first collection, when the samples were transferred to a refrigerator, where they were stored for 12h at approximately +2C. Following an equilibration period of 3h at room temperature, each, apart from one root sample from each site which was kept as an endogenous ethylene control, was innoculated with 2.5ml of acetylene. The subsequent assay procedure was identical to that described above. Calibration gases were checked on a more sophisticated GC (Hewlett Packard 5830A, by courtesy of the Dept. of Plant Science). In order to standardize the results, the root tissue in each experiment was separated from the adhering soil, both entities oven dried at 70C, and the dry weights obtained by weighing on a Mettler H10 analytic balance.

III. Results.

A summary of the results to the experiments described above is presented in this chapter. Interpretation of these is left for the Discussion in Chapter IV. In all figures (with the exception of Fig. 11), where plotted points represent the mean of a data set, the vertical intervals define the 95% confidence limits as determined by the Student's t-test.

A. Cold adaptedness.

1. Leaf tissue.

Although V. adunca is not a wintergreen species at either ploidy level under normal circumstances, it displayed via the cold hardiness tests, a remarkable degree of hardening ability in the leaf tissue. In Table 2, a summary of these results, the temperatures estimated to be representative of LD-50 values, range from -5C to -27C, the latter value having been obtained with plants subjected to cool temperatures and short days. Others viz. D, exposed to shortening days in a standard greenhouse, but with no reduction in ambient air temperature, also indicated a hardening of their older leaves, but equivocal change in the hardiness of younger leaves. Plants grown out of doors were less hardy than expected in August, but more so in September (F & G), thereby highlighting the plasticity of V. adunca, and frustrating the experimental design.

From the summarized results, a sample set of differences between matched pairs was analysed statistically

Table 2. A summary of LD-50 cold hardiness values for the leaf tissue of mature Viola adunca plants subjected to different growing regimes. Abbreviations are:GH = standard greenhouse; CEC = controlled environment chamber; Trop-arctic = cool greenhouse. (For details of the facilities used, see Appendix II).

	Cold hardiness (C)			
	2N		4N	
Conditioning	young	mature	young	mature
A. GH; mid-April; natural light. (start of CEC).	-	-12.5	-	-10.0
B. CEC; 4weeks SD; cooling temps.; night frost.	-	-16.0	-	-18.5
C. CEC; 2 weeks LD; warm temps.	-7.0	-7.0	-5.0	-5.0
D. GH; late-Nov.; natural light.	-10.0	<-15	-7.3	<-15
E. Trop-arctic (4 weeks);mid-Dec; natural light.	-18.3	-26.7	-20.0	-17.8
F. Outdoors; mid-August. -	-	>-6.0	-	>-6.0
G. Outdoors; late-September.	<-15	<-15	<-15	<-15

(Table 3), with the null hypothesis that the mean difference was not different from zero. This hypothesis could not be rejected at an 80% level, implying that the tests were unable to resolve with any reasonable degree of certainty, a difference between ploidy levels within the context of the experiment.

2. Water relations during hardening.

During the course of the hardening effected in the CEC (A and B in Table 2 represent start and finish respectively), leaf water potentials were obtained, and their components established as described in Methods and Materials. These results are represented in graphic form in Fig.10. One of the striking, although not surprising features of this, is the overall similarity in water potential values between the ploidy levels. The other noticeable feature is the distinct and statisticallly significant (98% by the 2-sample t-test) decrease in the (osmotic + matric) component over the four-and-a-half weeks of hardening[6].

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[6] (The change in the osmotic and matric components over the hardening period, together with the relatively stable total water potential, implies an increasing turgor. This is contrary to the observations of Wilkinson (1977) who found that turgor decreased during the hardening of Ledum groenlandicum. This result is likely to be at least partially due to a difference in cell wall elasticity.

Table 3. A comparison of matched pairs from Table 1, together with the statistics which permit the statement that there is no overall difference in cold hardiness of mature plant leaf tissue between chromosome races. This null hypothesis, according to Student's t-test, cannot be rejected at anything above the 80% confidence level.

Hardiness (-C)		
2N	4N	difference
18.3	20.0	-1.7
26.7	17.8	8.9
10.0	7.3	2.7
7.0	5.0	2.0
7.0	5.0	2.0
12.5	10.0	2.5
16.0	18.5	-2.5

mean difference= 1.99

std. deviation= 3.70

degrees of freedom= 6

observed .t= 1.4186

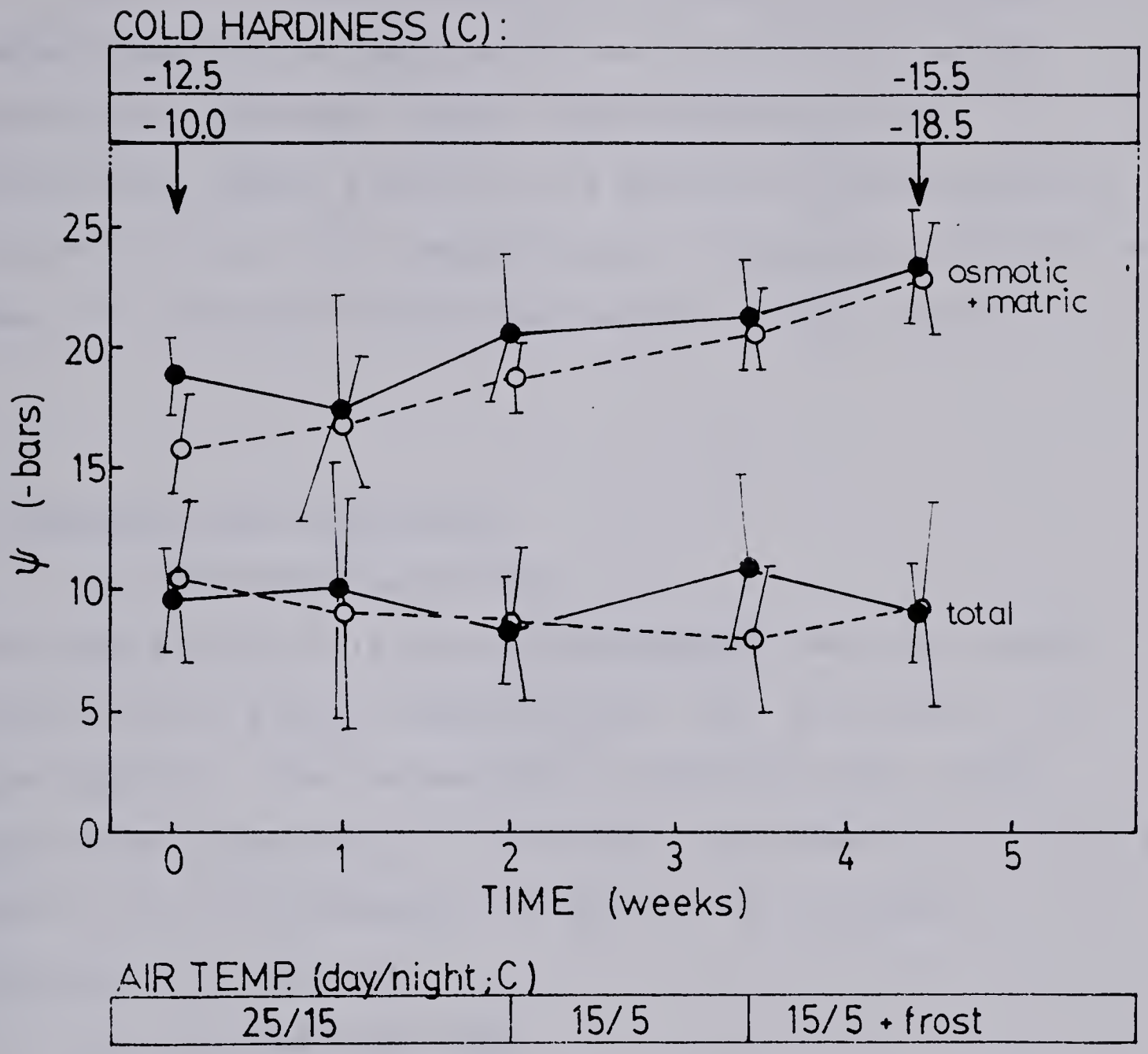

Fig. 10. Leaf water potential values determined psychrometrically over a period of hardening (●=diploid and o=tetraploid). The temperature regime is given beneath, and the associated leaf tissue cold hardiness above the plot. Vertical bars indicate the 95% confidence interval.

3. Seed cold hardiness.

Figs. 11, 12 and 13 show the effects of stressing temperatures on the germination success of incipiently germinating (imbibed) seeds. The influence of the stratifying regime's severity is distinctly discernible by the shift in survival temperatures, although no claim can be made for a distinct difference between ploidy levels.

4. Seedling cold hardiness.

a). Unhardened seedlings.

The single test on 14 day-old unhardened seedlings showed survival at a stress temperature of -6C, but none at -12C (see Table 4). Also noteworthy is the fact that the 2N population showed only 53% survival, as compared to the 4N's 100% at -6C, a difference not able to be validated statistically, however.

b). Hardened seedlings.

The combined effects of age and exposure to a hardening regime can be seen in the results obtained for the 6-8 week-old hardened seedlings (see Table 5). In comparing the two sets of results for the unhardened and hardened seedlings, it can be seen that at least 10C more of frost is able to be tolerated by the latter.

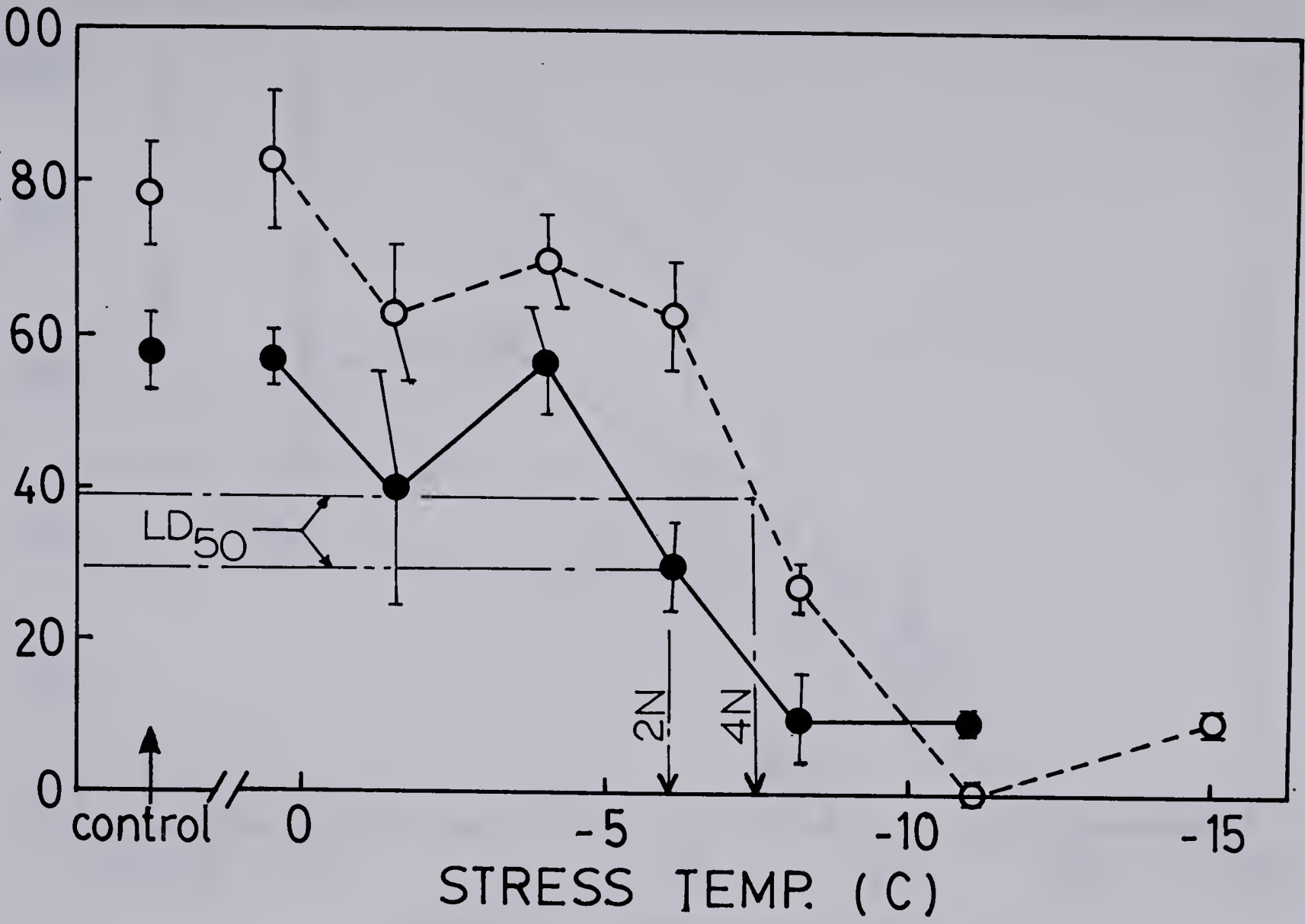

Fig. 11. Survival of cold stressed seeds with artificially broken seed-coats. (Note that the vertical bars represent the standard error of the mean of three sets of seeds, and not a 95% confidence interval as in all other figures). (●=diploid and o=tetraploid).

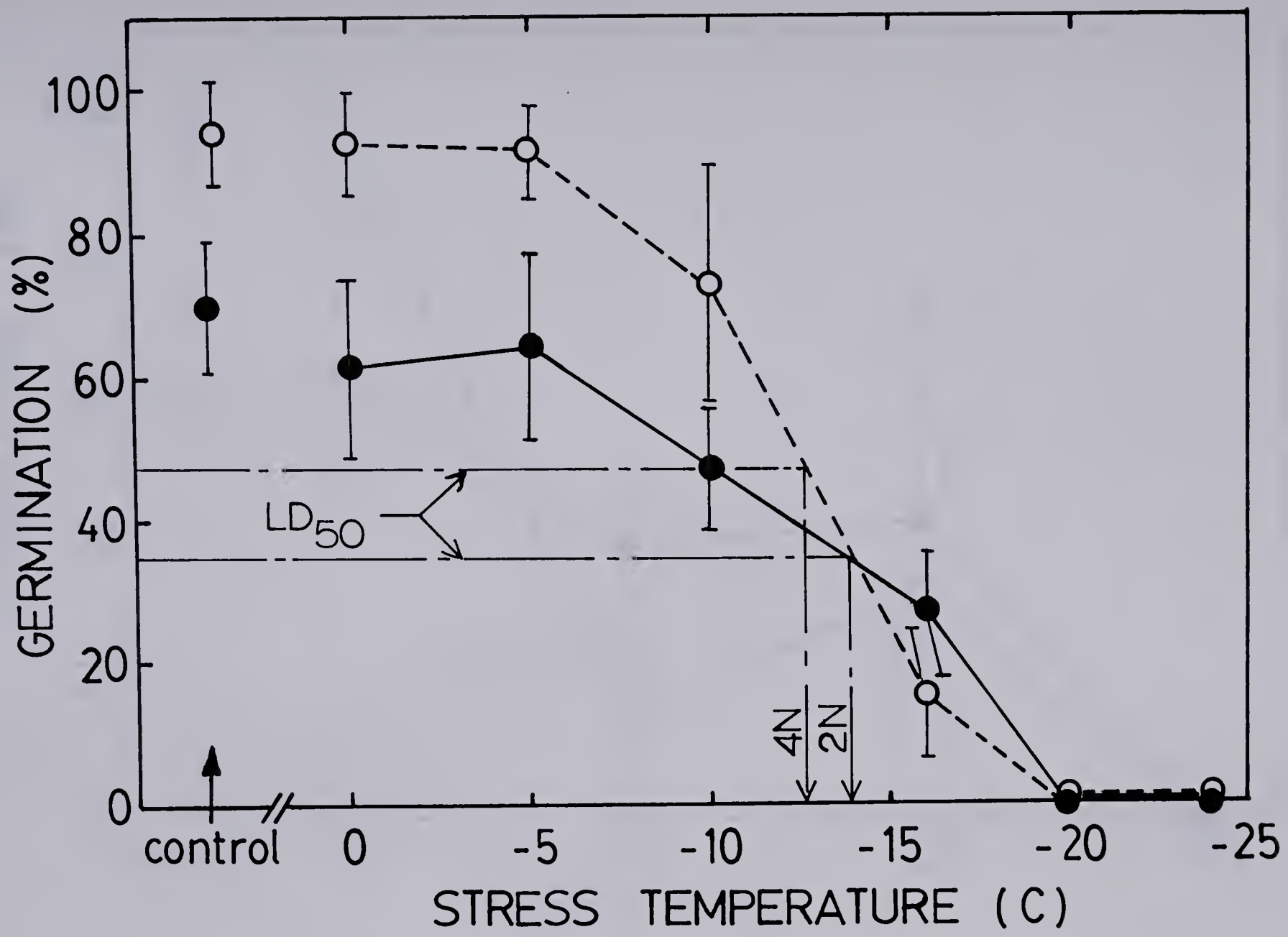

Fig. 12. Survival of cold stressed seeds subjected to stratification at low, but non-freezing temperatures. (●=diploid and o=tetraploid).
Vertical bars indicate the 95% confidence interval.

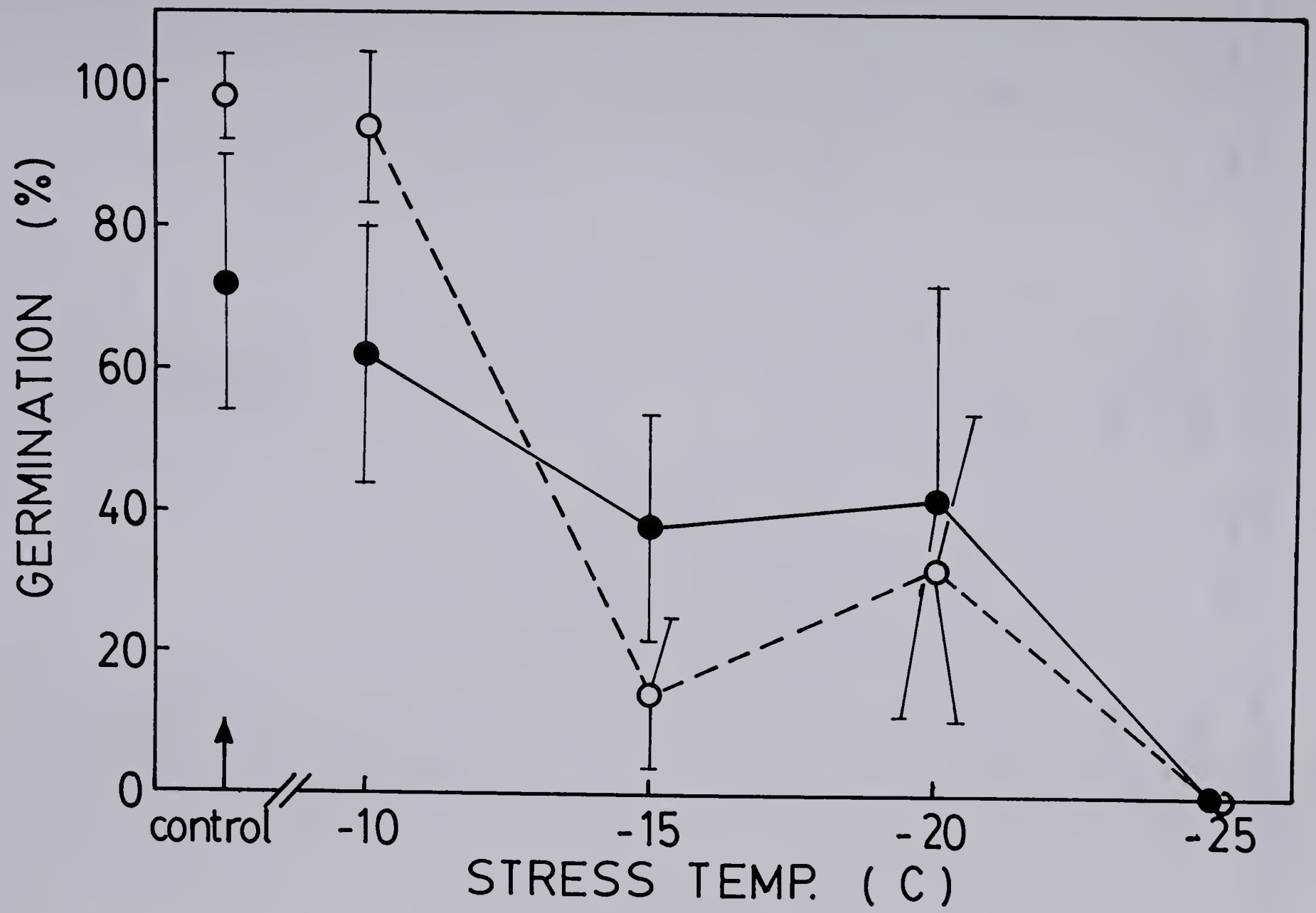

Fig. 13. Survival of cold stressed seeds subjected to stratification at low temperatures, including a period at sub-zero (●=diploid and o=tetraploid). Vertical bars indicate the 95% confidence interval.

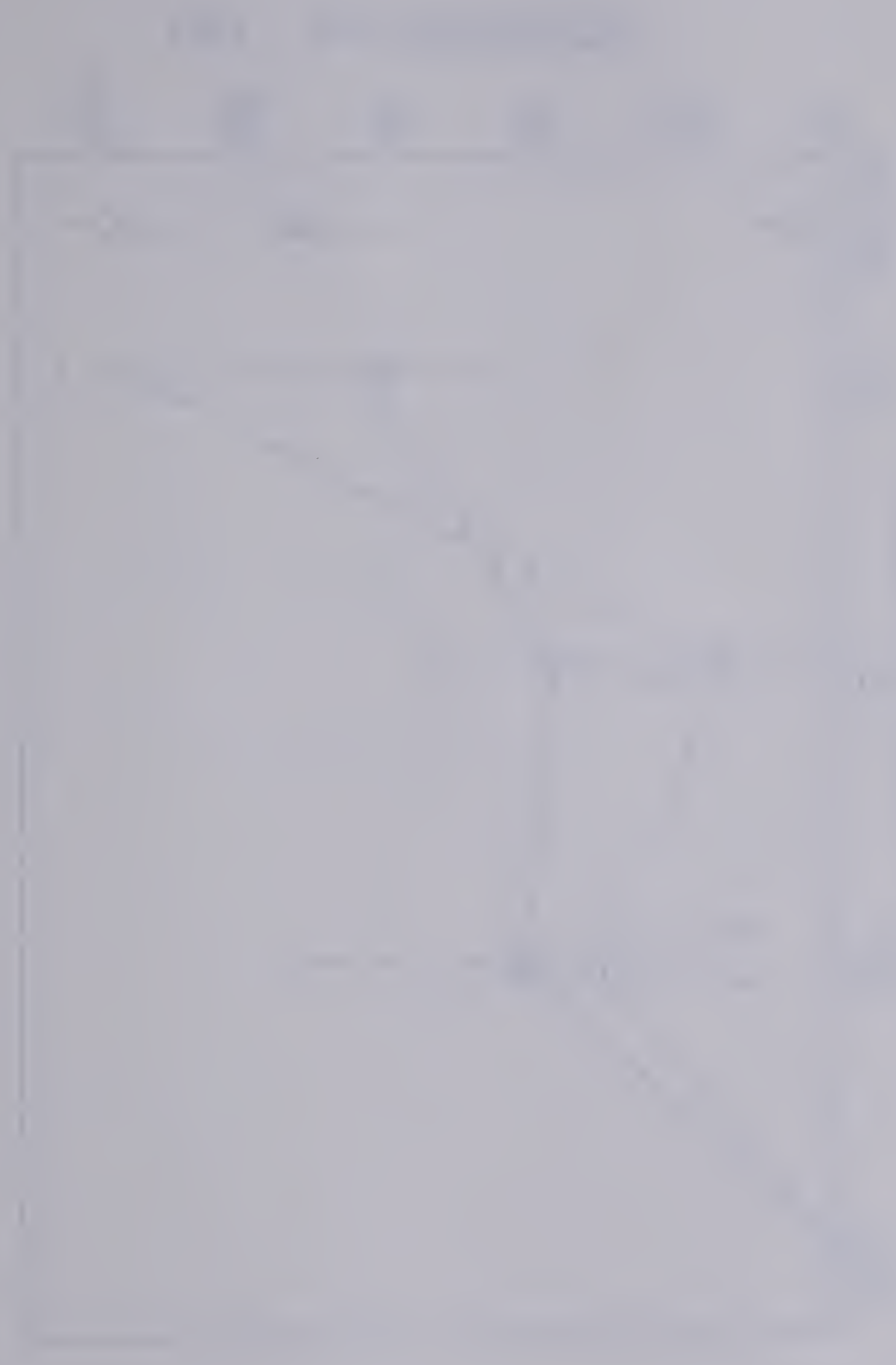

Table 4. Cold hardiness of unhardened seedlings 14 days after the initiation of germination. Most seedlings were in the early stages of production of the first true leaf. Figures represent mean percentages of 3 sets of 5 seedlings each.

Stress Temp. (C)	Survival (%) 2N	4N
0	100	100
-6	53	100
-12	0	0

Table 5. Cold hardiness of 7-8week old hardened seedlings. (See Methods and Materials for details of the hardening regime). Figures represent mean percentages of m petri dishes, each containing 9 seedlings.

Stress Temp. (C)	Ploidy 2N	4N	m
control	100	100	2
0	100	100	3
-5	100	100	3
-10	100	89	3
-16	100	100	2
-24	0	0	2

Table 6. Response of unhardened mature Viola adunca plants, 2 and 7 weeks after experimental cold stress, where n is the number of potted plants constituting the sample. Sample sizes are the same for both ploidy levels.

Temp. (C)	n	Response 2N	4N
0	3	No damage to any plants; still flourishing after 7 weeks.	As for 2N.
-5	3	At 14 days older leaves on all plants yellowing; young crown leaves maintaining a good green colour. One plant partially wilted. At 7 weeks, wilted plant now dead; other plants struggling with only two and four small new leaves respectively; all other tissue dead.	All aboveground shoot tissue quite dead.
-10	3	At 14 days; All above ground shoot tissue dead. No regeneration of shoot tissue after 7 weeks.	As for 2N.

Table 7. Cold hardiness of hardened mature plants at 2 and 7 weeks after stressing. Sample sizes are given by m and n for 2N and 4N samples respectively.

Temp. (C)	m	n	Response 2N	4N
con-trol	2	2	14 days: Good new growth of leaf tissue from crown.	Good new growth from crown and also trailing some shoots.
			7 weeks: All plants of both ploidy levels showing good growth.	
-10	3	3	14 days: As for control.	As for 2N for for 2 plants, some retarded growth on 3rd.
			7 weeks: All plants of both ploidy levels doing well with good shoot growth.	
-15	3	2	14 days: For both ploidy levels: crown area looks turgid and viable, but growth slow relative to -10C samples.	
			7 weeks: In 2 plants some stunted growth leaf tissue,but crown decomposed and these shoots not rooted well. Well developed leaves and buds in well rooted 3rd plant	As for 2N, with one doing well, and the other poorly in this sample of 2.

...over

Table 7 continued....

-20	3	3	14 days: All above-ground quite dead. 7 weeks: One poorly rooted 4N plant showing stunted growth; all others dead.	As for 2N for 2 plants, but crown of 3rd turgid.
-25	2	1	14 days and 7 weeks: All above-ground tissue dead in both ploidy levels.	

5. Mature plant cold hardiness.

A detailed description of the conditions of the test plants is given in each of the Tables 6 and 7. In summary, it can be seen that unhardened plants either survived poorly, or did not survive at all when subjected to a -5C stress temperature. On the other hand, survival of plants hardened under an arbitrary regime of short days and cool temperatures, was good at -10C, and poor at -15C.

6. Outdoor plants.

Although no quantitative results were obtained for the cold hardiness of plants exposed to the natural elements, it is evident that both races are, in a total sense, extremely hardy. All of the plants of both popultions were able to regenerate new growth in the spring/early summer of 1979, after a winter when the minimum temperature recorded at ground level was -32C. Due to the shallow embedding of the pots (approximately 5cm maximum) in the peat, it is likely that the entire plant, including the root mass, was exposed to a temperature close to this. (The mode of regeneration is discussed below under Productivity - see also Fig. 20).

B. Productivity.

1. Seed production through cleistogamy.

Collection of ripe seed capsules from outdoor plants during the late summer, and fall of 1978, revealed that 4N

plants were producing more seeds than their 2N counterparts. The seeds collected were counted, and data are presented in Fig.14, normalized with respect to the number of plants and time. In a final analysis, however, it was found that approx. 55% of the capsules in each population had dehisced before capture, thus making Fig.14 an underestimate of absolute seed production, but not upsetting it as a comparative measure. An analysis of the differences between paired weekly figures, allows rejection at the 99% level of the null hypothesis that the mean of these differences is zero. This allows the conclusion, that on a weekly basis, the 4N was more productive than the 2N.

The second and more rigorous investigation of seed production was that executed under controlled environmental conditions (see Methods and Materials), and yielded the data presented in Fig.15. This time the escaped seeds are included, empty capsules having been picked simultaneously with the ripe ones, and an average estimate added in for the number missing. Statistical analysis of the paired data points, as for the outdoor plants, reveals that the mean weekly productivity of the 4N plants is significantly greater than that for 2N plants. This statement is made on the grounds that the weekly difference between ploidy levels in the amount of seed produced per plant per week, is greater than zero at a 99% level ($t=5.7$ with 6 degrees of freedom).

Due to the fact that capsules were allowed to dehisce

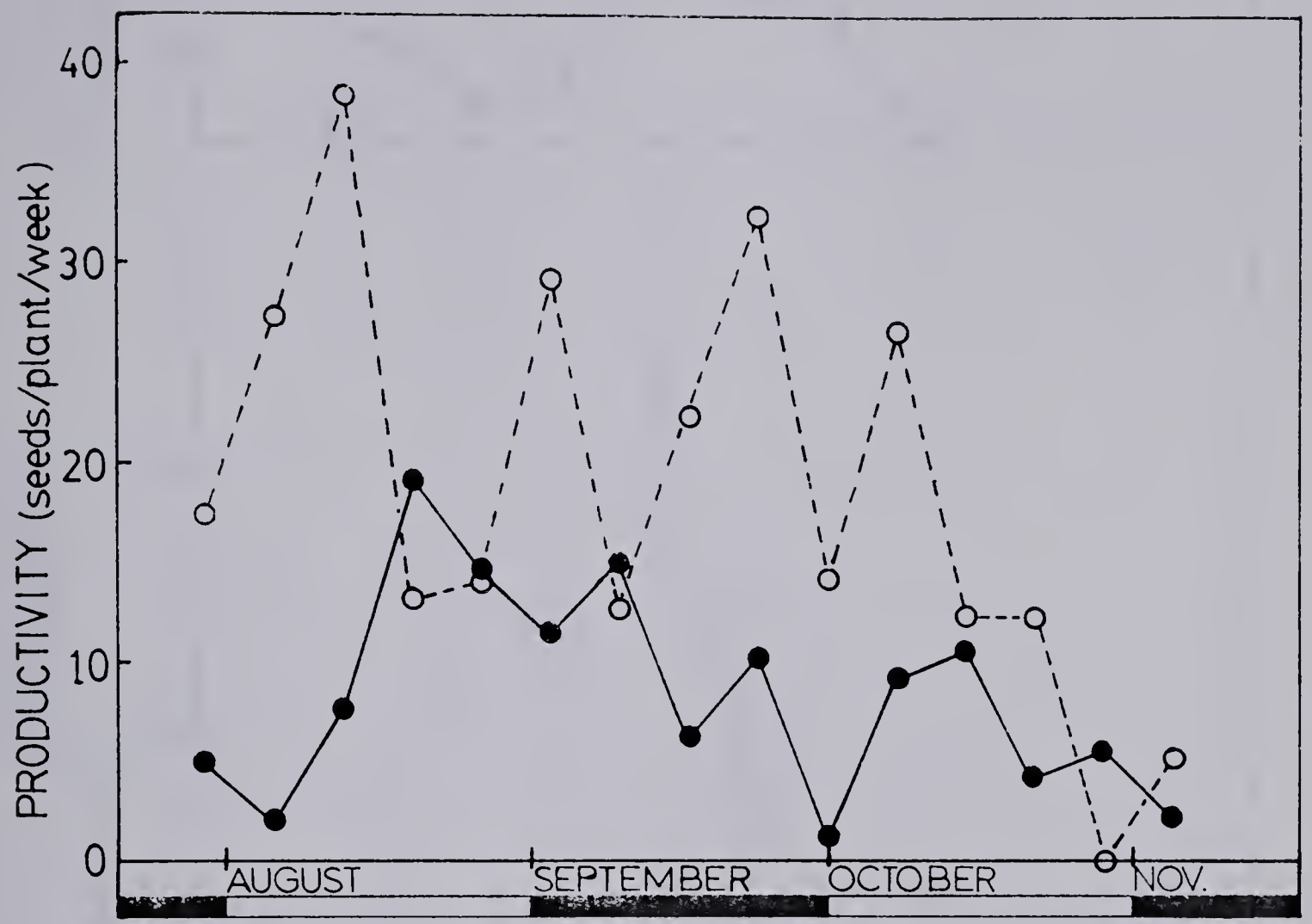

Fig. 14. Seed production of outdoor plants during the summer and fall of 1978. Owing to the number of seeds escaping capture, it is estimated that the quantities above are representative of only 45% of the true productivity (●=diploid and o=tetraploid).

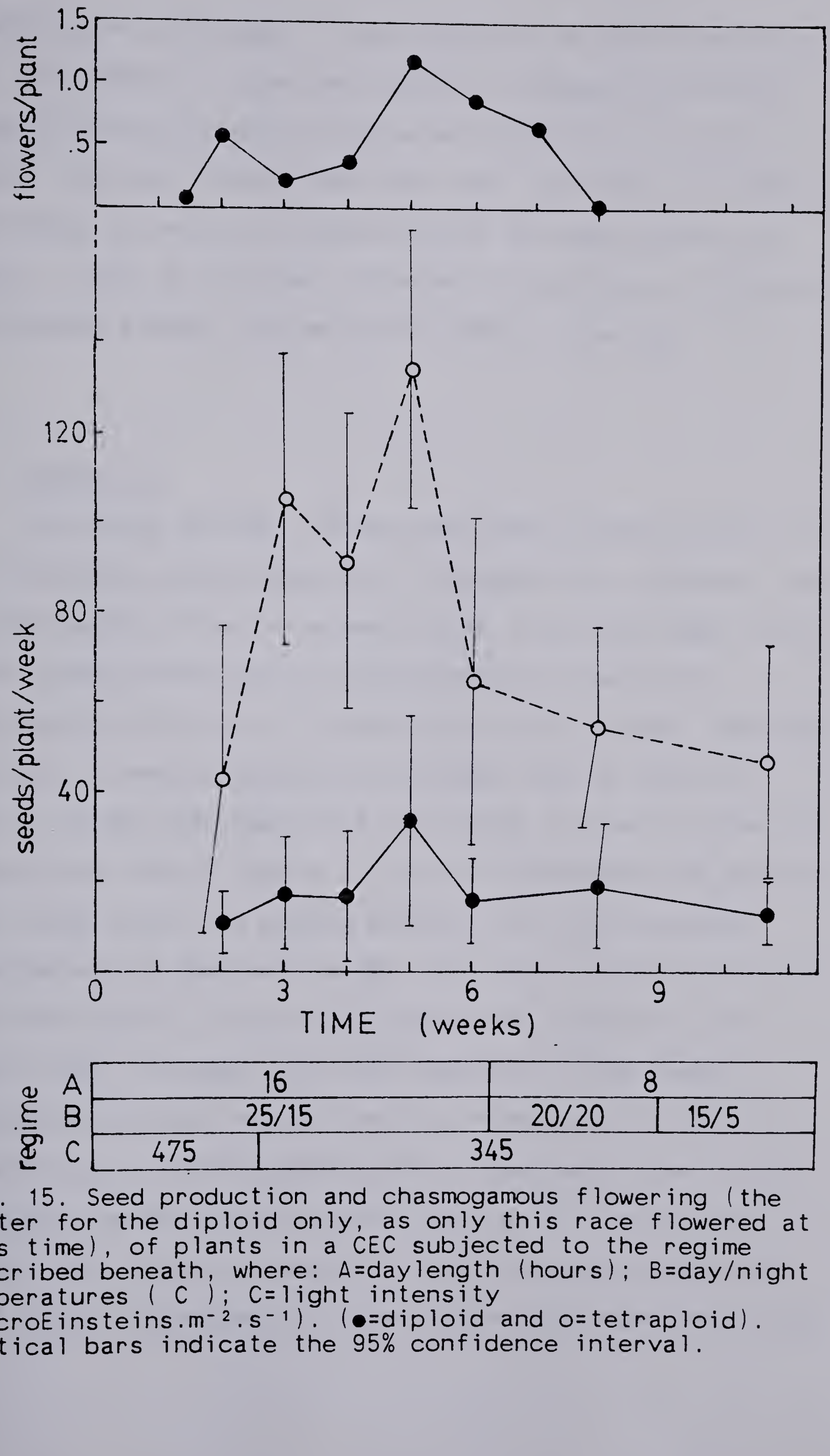

Fig. 15. Seed production and chasmogamous flowering (the latter for the diploid only, as only this race flowered at this time), of plants in a CEC subjected to the regime described beneath, where: A=daylength (hours); B=day/night temperatures (C); C=light intensity (microEinsteins.$m^{-2}.s^{-1}$). (●=diploid and o=tetraploid). Vertical bars indicate the 95% confidence interval.

inside paper envelopes, it was possible to obtain estimates for the number of seeds per capsule. Although occasional observations were made of capsules containing up to 30 seeds, the usual number was much less than this. In Table 8 the means of values corresponding to the data points in Figs. 14 and 15 are given, showing a significant difference in outdoor plants, but not so in those in the CEC.

1a. Flowering.

The above section, as was mentioned, refers solely to cleistogamous seed production. Although often observed, open (chasmogamous) flowers seemed rarely to produce seed, even when cross pollination was encouraged by simulating pollinator activity with camel-hair paint brushes. One very distinct flowering response was noted, and is shown in Fig.15. Under the conditions described, it was only the 2N population which flowered. It should be pointed out as well, that these were late season plants, and that the same phenomenon was observed in the late season plants of the CEC run referred to in Fig.10, although the latter was not quantified. Although both were apparently late season responses to being moved from the greenhouse to a CEC, it should not be attributed directly to daylength, as in one instance the daylength was 8h (see Fig. 10), and in the other, 16h. Other enviromental varialbles were comparable. Late season chasmogamous flowering was also observed on two

Table 8. The mean number of seeds per capsule for collections made during the productivity study. Also shown is the standard deviation (SD), and the sample size (n). In the comparison of means for ploidy levels, outdoor populations were different at a 99.9% level, whereas a null hypothesis claiming no difference could not be rejected at the 80% level for the CEC plants.

	2N			4N		
	mean	SD	n	mean	SD	n
Outdoor plants	13.6	3.2	15	17.2	2.4	14
CEC plants	14.5	4.4	7	16.5	1.9	7

occasions in field plants in their natural enviroment, once in a population most probably tetraploid[7]. Spring flowering, the norm associated with violets, was a little more equitably shared between the two chromosome races, although, as Fig.16 shows, the 2N was distinctly more prolific in the early days after removal from their cold period. In spite of the simulated pollinator activity, the chasmogamous flowers produced relatively few seeds, and a high proportion of wholly abortive capsules (see Table 9).

2. Germination.

During the course of experimentation, data were gathered concerning mainly the effects of cold stress on the germinability of seeds pretreated in various fashions. These are dealt with above in Figs. 11, 12, and 13. The controls, together with preliminary results, however, were useful in compiling a comparison between the effects of the treatments themselves. This composite analysis is presented in Table 10, together with the statistics of comparison in Table 11. The points which emerge from this are:

a. that seed coat integrity is highly influential in the supression of germination for both races; and

b. that extended low temperature treatment after imbibition significantly promoted germination.

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[7] One member of this northeast Albertan population (Richardson Fire Tower) gave a root tip chromosome count of 40.

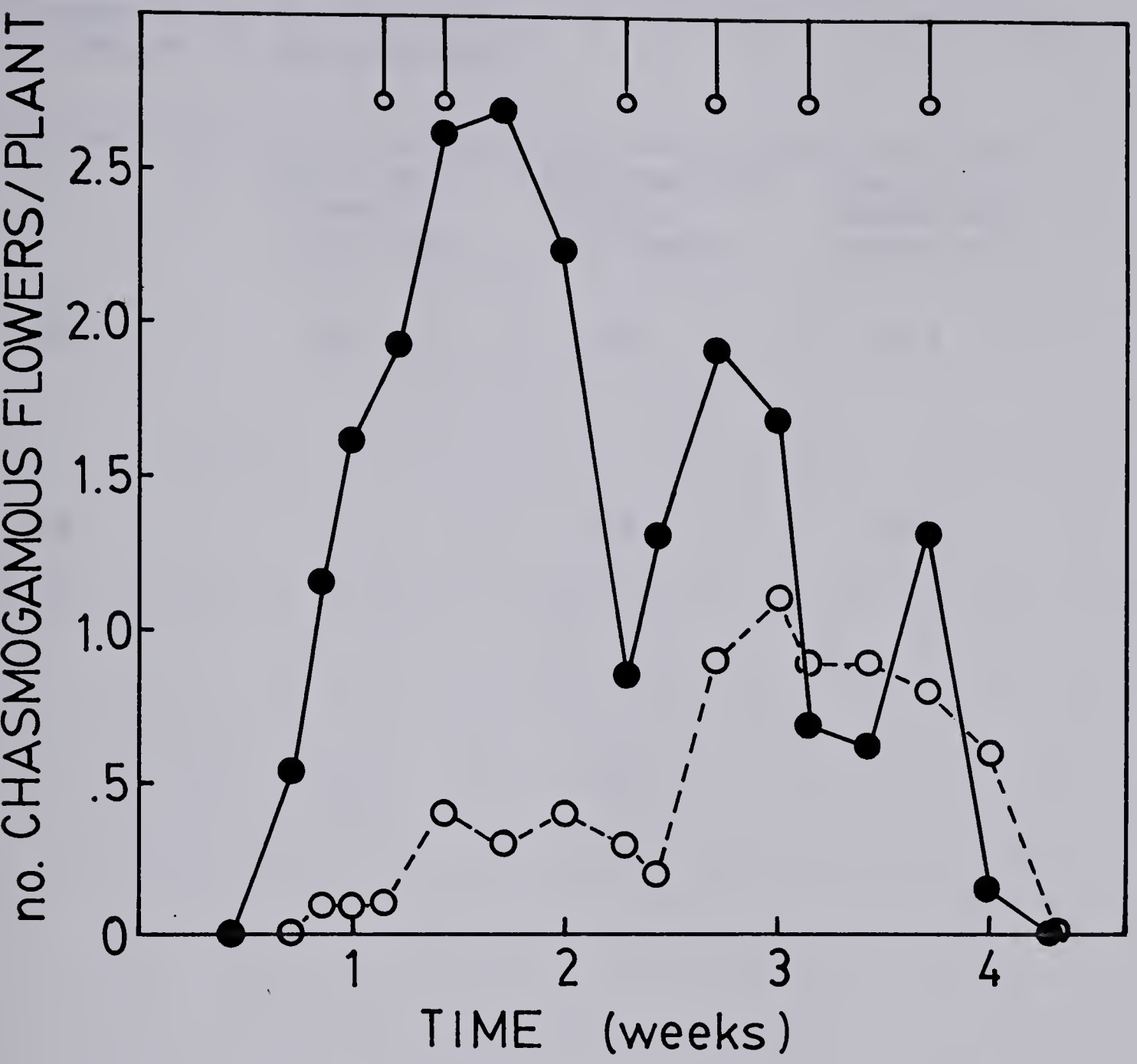

Fig. 16. Chasmogamous (open) flowering in the time following the removal of plants from a mild simulated dormancy period. The marks at the top of the figure represent artificial cross-pollination events (●=diploid and o=tetraploid).

Table 9. The fruit produced by the chasmogamous flowers described in Fig. 12. It should be noted however, that although cross-pollination was attempted, it is not known to what degree it was achieved.

	Total no. flowers. in period.	No. abortive flowers (nil seeds).	Ave. no. seeds per capsule.
2N	64	23	8.0
4N	14	8	6.3

Table 10. Summary of germination results. Treatments of the seeds were standardised with respect to post-ripening, scarification and imbibition.
Treatment variations were:
1. I. Mild stratification for 3 weeks;
2. II. As for I, but seed coats cut;
3. III. More severe stratification.
(For details see Methods and Materials on page 31).

	Ploidy	Germination success Mean(%)	Std.Dev.(%)	n	treatment
A.	2N	4.0	5.9	5	I
B.	4N	45.3	16.6	5	I
C.	2N	58.0	11.7	6	II
D.	4N	78.0	16.0	6	II
E.	2N	70.0	10.0	7	III
F.	4N	94.3	7.9	7	III

Table 11. Comparison of sample means in Table 10 using the t-test. The null hypothesis is that the means are equal, and the below table shows the degree of probability with which we are able to reject this hypothesis according to the code: 0 = unable to reject at the 90% level; 1 = can reject at the 95% level, but not at 98%; 2 = reject at the 99.9% level.

	A	B	C	D	E	F
A	-	2	2	-	2	-
B		-	-	2	-	2
C			-	1	0	-
D				-	-	1
E					-	2
F						-

When comparing the 2 chromosome races, it can also be noted that the 4N has a significantly higher germination success than the 2N for all treatments. Fig. 17 is a dynamic look at germination of the mildly stratified seeds (A and B in Table 10), whose germination success was measured after 14 days. At this stage seeds were removed individually, and their seed-coats cut (as described in Methods and Materials) with a razor blade. The reulting release of germination potential is very explicitly displayed.

In the tests involving extended stratification, it is difficult to separate this effect from the vernalizing effect of the cold treatment, because a large number of the seeds, especially amongst the 4N's, had imbibed so much over this period, that the swollen endosperm had split the seed coat longitudinally.

3. Seedling productivity.

The ability of germinants to gain a foothold in a competitive environments was considered as a possible point of difference between the two races, one of which is ostensibly a better colonizer. A crude measure of this was to determine biomass production under non-competitive conditions. Once again working with seedlings germinated from cleistogamously produced seed, the outcome of this experiment is shown in Table 12. With only two points in time, it is not possible to say whether or not the 2N

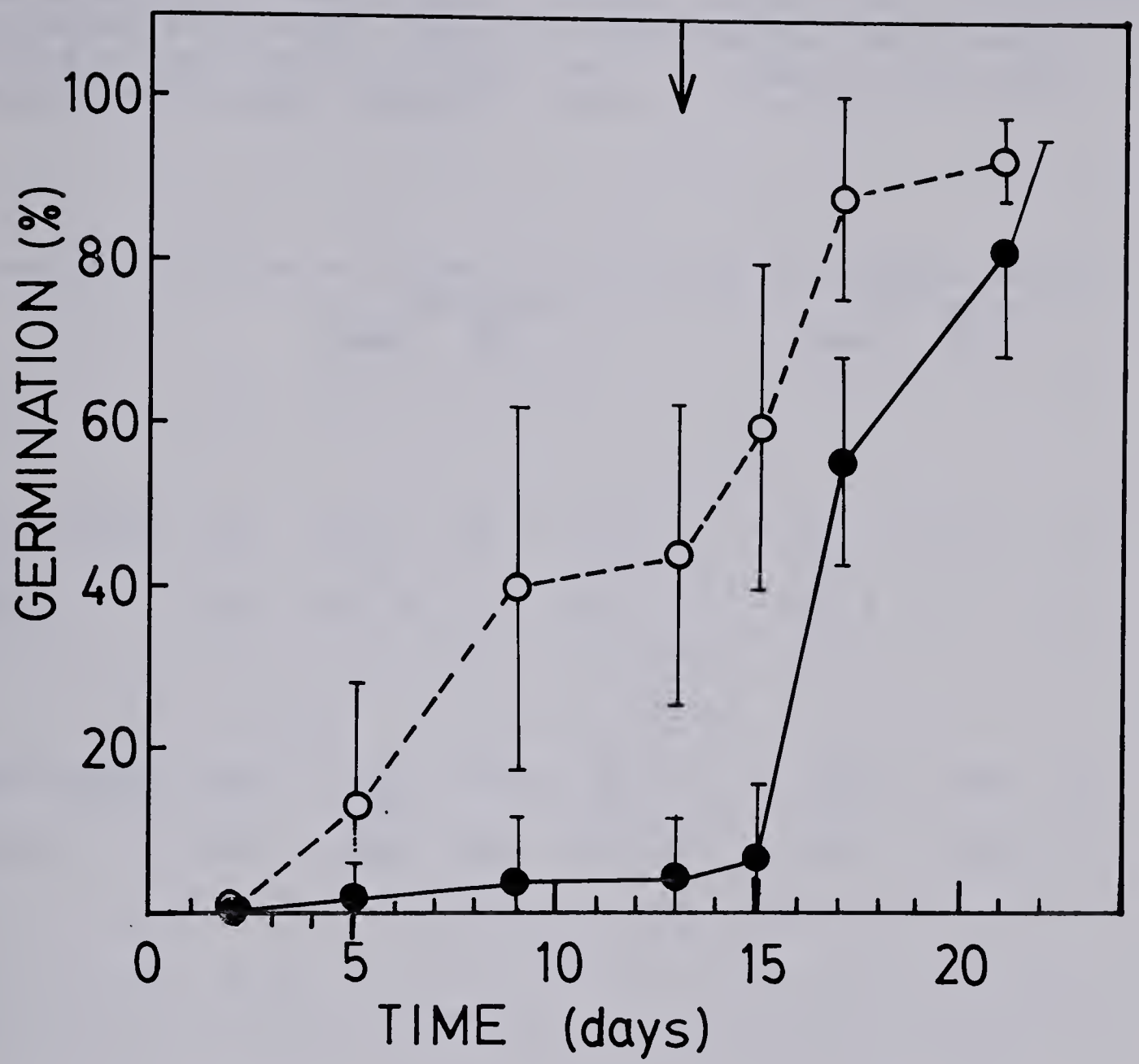

Fig. 17. Germination of mildly stratified seeds. The arrow indicates a cutting of the seed-coats of individual seeds with a razor blade (●=diploid and o=tetraploid). Vertical bars indicate the 95% confidence interval.

Table 12. Productivity in mg dry weight, and root:shoot ratios, of seedlings at 50 and 57 days after transplanting (as 14 day-old germinants) to a soil substrate (see methods and Materials: Productivity). Means are all significantly different at the 99% level, as computed by the t-test (*), or the Fisher-Behrens d-test (**) for populations with unequal variances (Campbell, 1974).

		50 days			57 days		
		mean	SD	n	mean	SD	n
Total prod. (mg).	2N:	62.2 *	35.0	9	81.1 *	45.4	18
	4N:	122.0	21.2	12	153.3	47.7	22
Root:shoot ratio.	2N:	.309 **	.128	9	.339 *	.086	18
	4N:	.469	.061	12	.401	.081	22

catches up to the 4N in biomass produced, but it is seen that it lags significantly behind at both 50 and 57 days under the conditions described. Also of interest is the fact that the biomass produced is directed towards the roots to a greater extent in the 4N than in the 2N, as is reflected by the significantly higher root:shoot ratios for the former.

Results for the experiment designed to investigate the effects of temperature on net productivity in the early stages of seedling growth are summarized in Fig 18. Due to the high mortality rate and consequently small sample sizes (per cent survival in the 12 seedling sample is given in the figure), variability is high in places[8], and no significant differences between races can be shown. Even comparison of the extreme points is hindered by this variability in the case of the 2N, although the 4N's in the warm house are significantly more productive (at the 95% level) over 5 weeks, than those in the cold room. The distribution within the plant of the tissue amassed is displayed in the root:shoot ratios of Fig. 19.

4. Vegetative regeneration.

The phenomenon of plantlet formation, its nature ,its occurrence and its possible role, are described elsewhere in this thesis. However, for completeness in the discussion of

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[8] High variability, especially at the higher temperatures, is most probably the result of pathogen activity, which appeared to be the cause of high mortality rates.

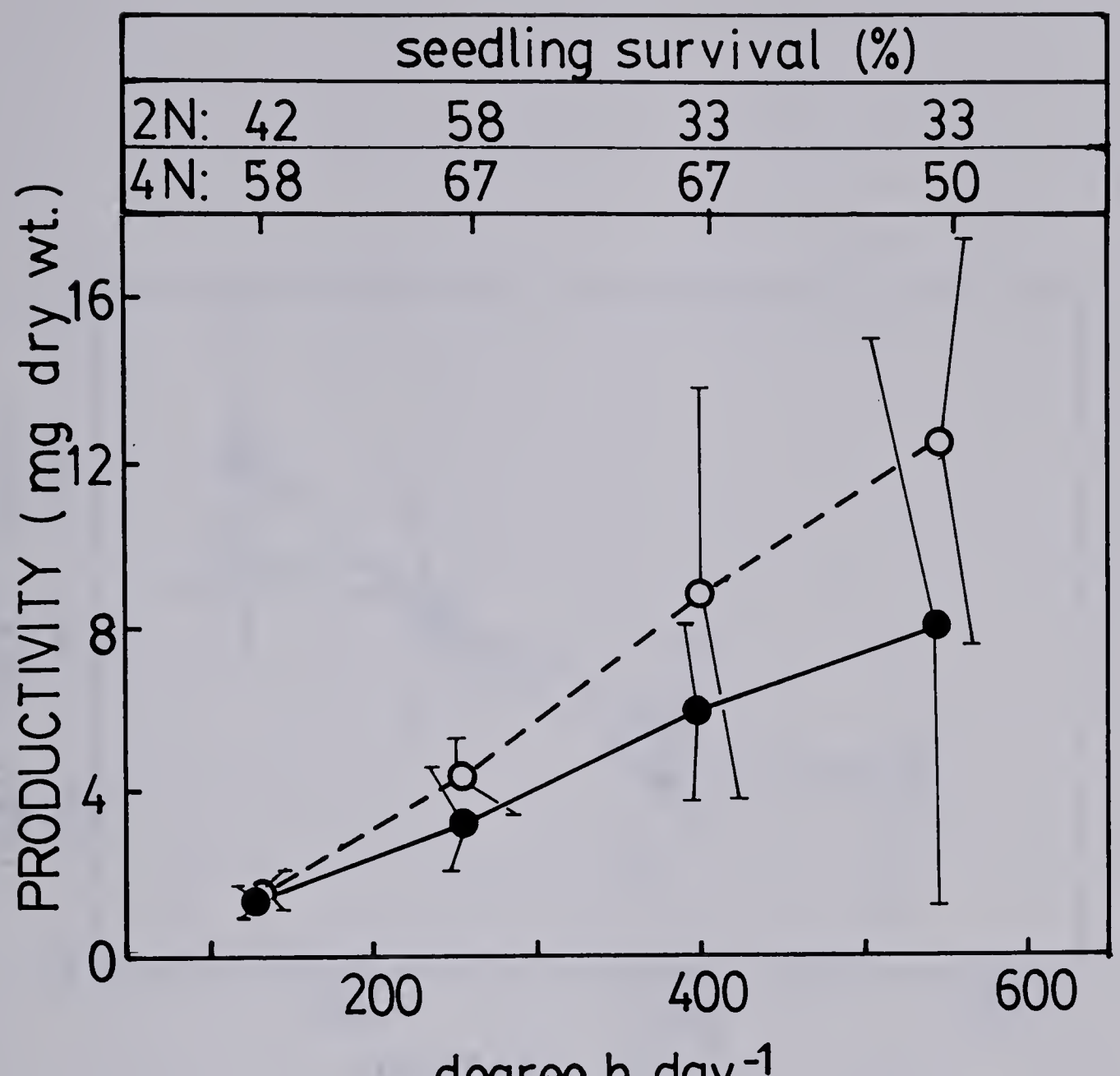

Fig. 18. Total dry weight production in 5 weeks by seedlings subjected to different temperature regimes (see Methods and Materials for details; ●=diploid and o=tetraploid). Vertical bars indicate the 95% confidence interval.

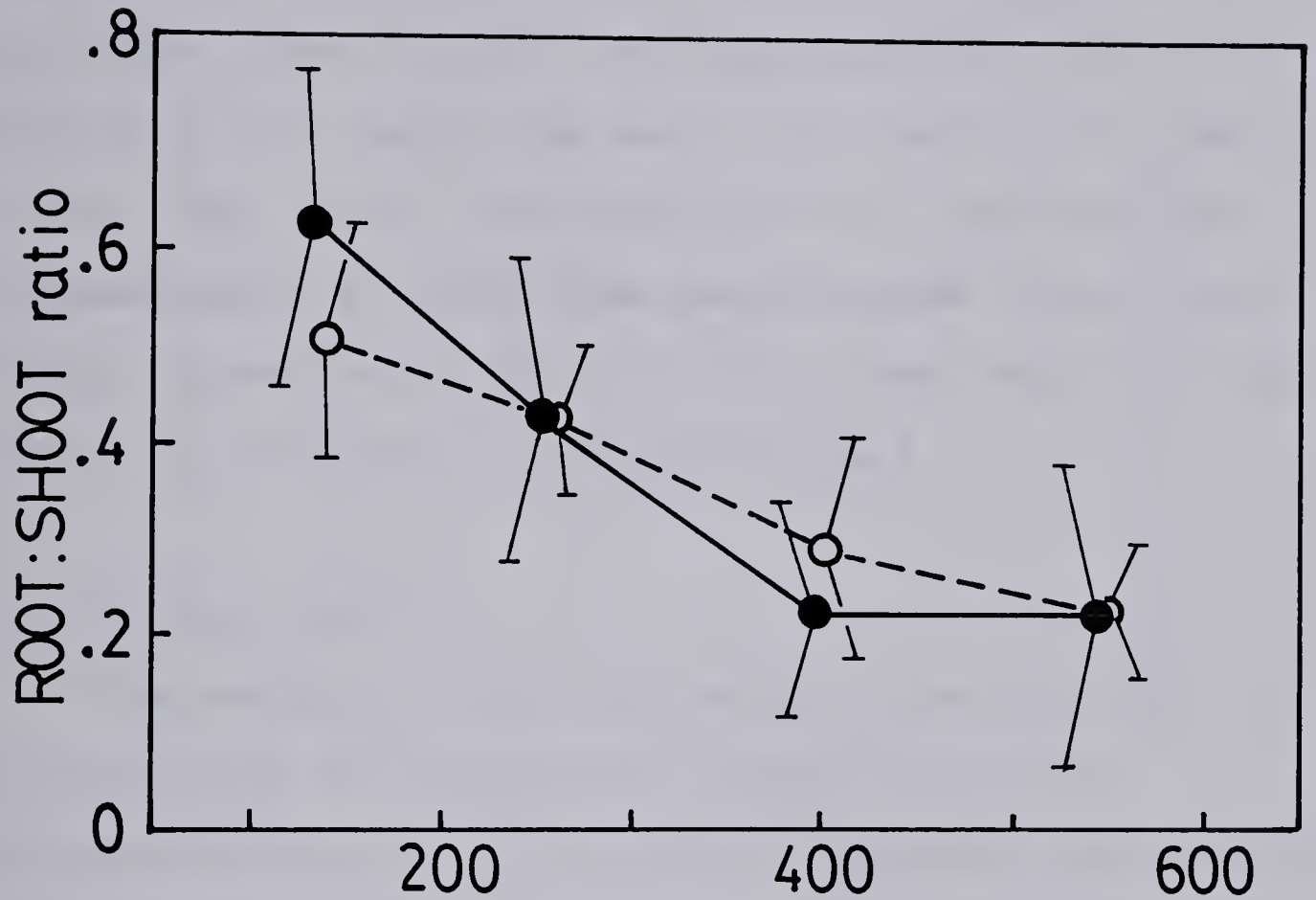

Fig. 19. Root:shoot ratios of the plants referred to in Fig. 18. (●=diploid and o=tetraploid). Vertical bars indicate the 95% confidence interval.

productivity, it is worth noting how this process manifests itself in time. Based on the overwintered outdoor plants, spring emergence (in the late spring of 1979) of plantlets regenerated from the previous year's root system was monitored. (See Fig.20). The advancedness of the 2N population can readily be seen, until early July when it was evident that all of the plants survived, and each was represented by at least some above-ground tissue, even though in many cases the plantlets formed were no longer materially attached to the parent plant.

C. Nitrogen fixation.

The results of this preliminary investigation into the possible role of rhizospheric associations with nitrogen-fixing bacteria in the differential distribution of the chromosome races, are summarized in Tables 13 and 14. In brief it might be stated that both races, as interpreted from the results of the GC survey, have representatives capable of establishing the necessary nitrogen-fixing associations under at least some conditions. (The measure of nitrogen-fixing ability in this section has been left as the amount of ethylene produced with respect to the mass of root tissue used, and time. No attempt has been made to relate this to nitrogenase-activity <u>per se</u>). As can be seen from the invariably low activity in soil samples however, it can be stated with certainty that fixation is associated with the rhizosphere.

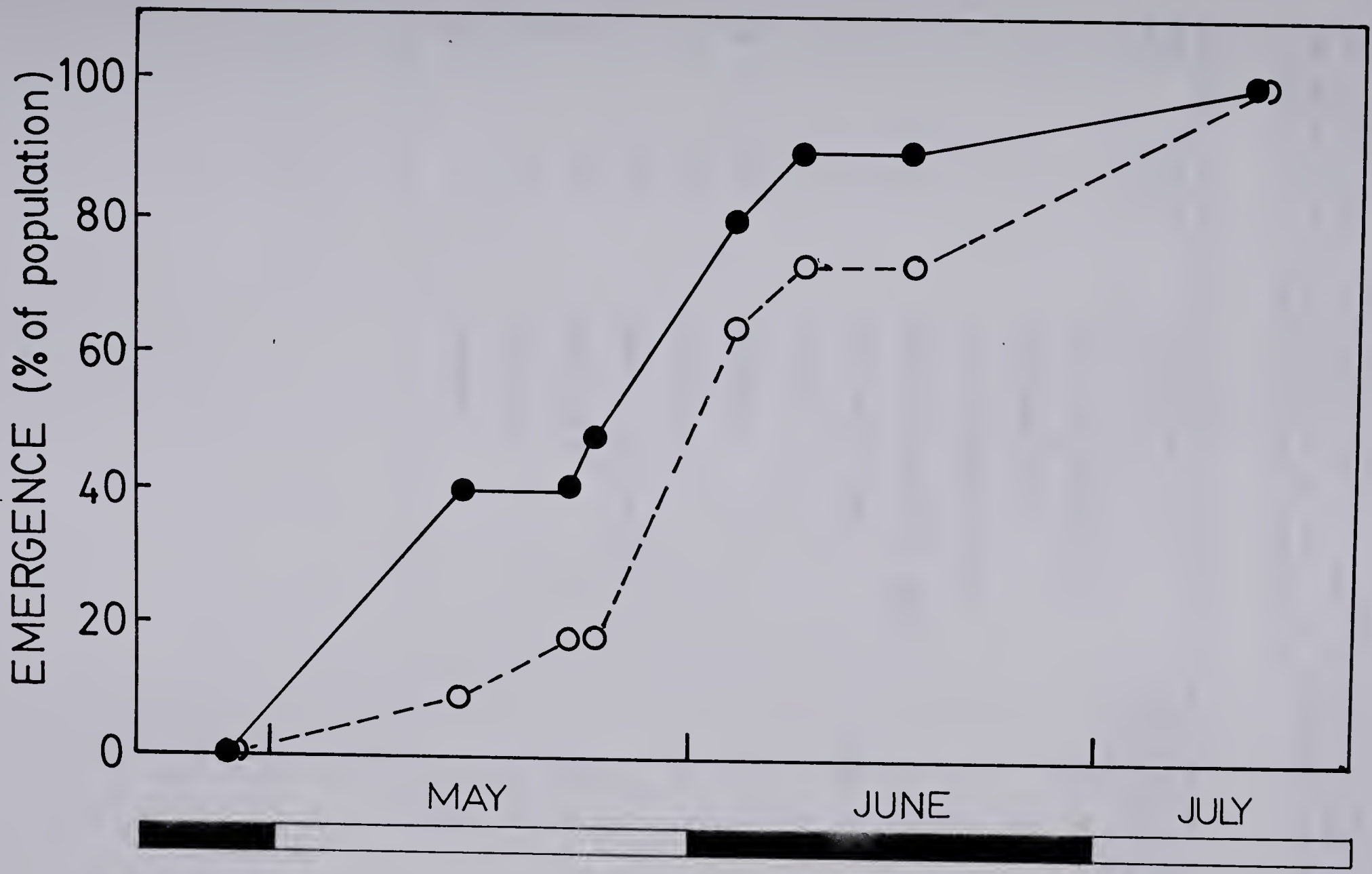

Fig. 20. Spring emergence of shoot tissue after the winter of 1978/1979, during which a minimum ground surface temperature of -32C was recorded (●=diploid and o=tetraploid).

Table 13. Ethylene production (nanomoles.(g root dry wt)$^{-1}$.hour^{-1}.) for root samples from plants brought back to the laboratory in August 1978. See Fig. 9 on page 42 for the approximate collection sites, and Methods and Materials for details of sampling and processing.

map ref.	ploidy (N)	Location	Ethylene prod. (see caption)
a	4	East of Redwater	96.2
b	4	Highway 643	1.3
c	4	Sterco coal mine	51.5
d	4	Rocky Mountain hse.	23.3
e	4	Big Horn Dam	423.5
f	4	Canal Flats	174.9
g	2	Coleman	119.9
h	2	Cranbrook	167.7
j	2	near Lundbreck	3.6
k	4	Woolford	98.5
m	2	Montana	12.8
n	2	Denver	4.8

Table 14. Ethylene production (nanomoles.(g root dry wt.)$^{-1}$.h^{-1}) in root samples collected in the field during June 1979.

Site	Control	Roots sample1	sample2	Soil
Cypress Hills:				
Reesor L.(2N)	0	23.3	0	0.5
Graburn (4N?)	0	0	11.7	2.0
Lodgepole(?)	-	4.4	6.4	0.2
Woolford (4N)	0	37.7	10.3	0.1
Lundbreck(2N)	0	0	13.2	0.1
Coleman(2N)	0	20.8	13.1	0
near Fernie(3N?)	0	0	29.2	0.3
Cranbrook(2N)	0	0	0	0.2
Canal Flats(4N)	0	8.0	0	0
Big Horn Dam(4N)	0	44.2	26.3	0.2
Rocky Mt. Hse.(4N)	0	12.8	13.2	2.3
Other plants:				
Arctostaphylos	-	16.7	38.7	0
Nodulated bean	-	171.4	-	-

In spite of the limitations of this indirect method in providing quantitative results, it is clear that fixation, where it occurs, is more pronounced in Table 13 than in Table 14. The former set of plants, it should be noted, were kept under greenhouse conditions for approximately 3 months, and kept well watered (with distilled water) until the time of sampling. Those in Table 14, on the other hand, were sampled directly in the field during the summer of 1979, when many of the sites were experiencing extremely dry conditions. This may point to an advantage which high soil moisture is able to offer the nitrogen-fixing microorganisms, and lead us to expect a positive correlation between nitrogen-fixing activity and soil water potential.

An investigation by light microscope of the rhizosphere biota of plants from Canal Flats and Rocky Mountain House suggested that the nitrogen-fixers were probably of the genus Clostridium.

IV. Discussion

Before attempting to interpret the experimental results of the last chapter, it might be useful to recapitulate the working hypotheses which formed the design skeleton. These were stated as being:

a. that the rigours of the northern latitudes represent a geographical threshold unable to be crossed by one of the races, but not the other; and

b. that the post glacial wasteland presented greater problems with regard to recolonization for one race than for the other.

Although severely limiting in their inflexible generality, they provided a useful framework for experimental design and the gathering of data. To some extent specific answers can be provided for those initial questions, but only in truths less than whole. Part of the reason for this is that these hypotheses are in ways inseparable, being testable only in terms of whole plants in relation to their environment, a complex whole which usully defies even partial characterization. by the handful of experimental facts established.

An analysis of the problem can best be achieved by invoking Hutchinson's (1958) concept of the niche as an n-dimensional hypervolume in n-dimensional space. However, in order to make any progress in the investigation of the nature of such an hypervolume, we need to reduce the number of varialbles sufficiently to be able to manipulate the

remainder with an acceptable amount of precision. And so, by fixing m dimensions, we are able to reduce our n-dimensional space to one of (n-m) dimensions[9]. The fixing is an intuitive process, and can in no way be achieved totally objectively. Only trial and error can tell us whether our choices have been justified, but that in turn constitutes an investigation of the very dimensions we were hoping to circumvent in order to make productive headway.

To illustrate this geometric analog, let us assume that we wish to partially characterize two volumes (n=3) which, unbeknown to us, are a sphere, and an ellipsoid of revolution about the major axis. If we conducted the investigation in two dimensions (m=3-2=1) by fixing one of the three Cartesian axes for each, we might see identical circles and conclude that the two volumes under investigation are identical - but this could only stand as a truth relative to our perspective. Should the static dimension be refixed such that our plane of inspection intersects at another point on the major axis of the ellipsoid, we could conclude (quite rightly) that, relative to our point of view, we are observing circles of different diameters. If, on the the other hand, we were to fix an axis parallel to the major axis of the ellipsoid, it would be quite plain that our objects of interest are of separate species viz. a circle and an ellipse.

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[9] The inherent weaknesses of objective scientific research lie ominously concealed behind the words "sufficiently", "acceptable", and "fixing", and can at best be tolerated with suspicion.

With extreme caution let us transfer our attention to Viola adunca. The investigation of the two chromosome races of the species may be considered as analogous to that of the sphere and the ellipsiod, with the exception that our unknown conceptual hypervolumes can only be described by probability clouds (much as the electron shell of an atom is described in the terms of Heisenberg uncertainty), rather than by definite geometric surfaces. But, propped up by the statistics of normality, we may proceed to regard the objects of our scrutuiny as well-defined (n-m)-dimensional volumes by fixing those m dimensions which we consider to be irrelevant to the study.

One of the first variables to have been fixed in the work with V. adunca, was that of geographical location. This was done by choosing to use plants collected solely within a small range, at a locality which is reported not to have been subject to glaciation during the Wisconsin viz. the Cypress Hills (Westgate, 1968). This choice assumes an intersection of both hypervolumes at points which allow for valid comparison, much as the intersections of sphere and ellipse were assumed to be orthogonal to the axes. Our correctness of choice is ultimately untestable, especially when based on an incomplete knowledge of geological and evolutionary events in the preceeding and intervening time. Having thus fixed some of the variables, Mauer (1977) proceeded to investigate the dimensions of net assimilation and water relations in mature plants (fixing the dimension

of phenology). His conclusion (see Table 1 on page 9.) was that he had chosen a perspective which revealed similar projections of the two hypervolumes, a null result. In the present study an attempt was made to obtain a different perspective, while holding to some of the assumptions made by Mauer.

The first attempt to extend the knowledge of V. adunca by the investigation of absolute cold hardiness (as opposed to environmental cold hardiness, a factor considered below) and water potential at different seasonal stages in mature plants appears to have led to a similar null point. Although by no means a dramatic conclusion, it provides at least partial grounds for the rejection of the "tolerance of extremes" viewpoint as applied to this species. (The only exception to this conclusion is the possible superiority of unhardened seedlings with regard to frost tolerance, as is shown in Table 4 on page 54., and discussed briefly below.)

In further shifting our observational standpoint so as to allow for an examination of aspects of productivity other than net assimilation in mature plants, some possible differences started to become apparent. The differences in cleistogamous seed production, seed viability and seedling tissue synthesis indicate a quantitative superiority of the 4N, whereas with respect to chasmogamous flower formation and regeneration of shoot tissue from overwintered roots, the 2N was more productive. The survey of rhizospheric nitrogenase activity once again revealed no distinct

differences between the ploidy levels, although it is of interest that this species is in some cases a symbiotic fixer. If our assumptions have been correct, and the choice of plant material from an area of sympatry in the Cypress Hills gives us an idea of the behaviour of refugial populations of both ploidy levels at a time when the ice sheet was starting its retreat, then we may construct a verbal model to describe the phenomenon of present day distribution. At best however, what we will have is the description of some of the processes which might have been involved in allowing the one chromosome race but not the other to occupy the northern part of the range, while preventing the reverse spread of the 4N into the more southern domain of the 2N. (See Fig. 1 on page 11 for a description of the present day distribution).

Based on the data available we must necessarily reject the first hypothesis that the 4N is hardier than the 2N when considering mature plants. (If anything, the reverse situation must be recognized, with the cushion-like growth of the 2N plants tending to protect the crown tissue from brief periods of frost). On the other hand, if we are to accept the hypothesis of Hall (1972) that, ploidy is related to the oxygen requirements of the roots, then there exists some explanation for the 4N being found only on drier sites, or alternatively the colder of the wet sites, the latter due to the higher solubility of oxygen at low temperatures. Although by no means a complete explanation in our case, it

is worthwhile to bear in mind that this proposed tolerance of an indirect water (excess) stress may be contributory to the overall scheme.

The idea that there might be a ploidy related difference in an ability to establish nitrogen-fixing associations also constitutes an hypothesis under the heading of stress tolerance viz. nutritional stress. The results show that indeed there are such associations which would benefit a species colonizing a poor substrate, but the fact that both races are implicated, once again causes us to reject the hypothesis as a key factor. It should be noted that this preliminary study is not wholly consistent with the main project, as it is a geographically wider survey and does not restrict itself to the area of sympatry, thus violating one of the rules governing our chosen perspective. Nevertheless its contribution to the picture can easily be recognised.

Turning our attention to the second hypothesis which regards colonization of the glaciated regions, a more likely set of contributory facts emerges. These facts are all associated with the seed and seedling stages, where the 4N appears to be at a distinct advantage over the 2N. By utilizing the efficient selfing mechanism of cleistogamy, the 4N is able to generate more propagules than its diploid counterpart, and these with significantly higher germinability. Once germinated, the established seedlings appear to be more productive as well. (Unhardened 4N

seedlings may also be marginally cold hardier than the 2N, as is shown in Table 4 on page 54, although this observation requires greater experimental resolution for a more positive assertion. An attribute such as this could provide 4N seedlings with a competitive edge over the 2N's during the spring when sporadic frosts are likely to occur.) If these are factors which occur in the natural enviroment, their combined effect could contribute significantly to the presence of the tetraploid in the recolonizing vegetation after the Wisconsin glaciation.

What then of the other observed differences? Flowering (chasmogamous) seems to be something more strongly associated with the 2N than with the 4N, at least for the conditions encountered by the experimental plants. Should this again be a feature of plants in their natural environment it would appear as though the event of polyploidy has been accompanied by a tendency away from outcrossing, and, as indicated above, towards cleistogamous selfing. In view of the simple models of the effects of outcrossing and selfing on diploid, autotetraploid and allotetraploid populations of different ploidy levels presented in Appendix IV, this is rather interesting. Comparing 2N and auto-4N for 2 alleles at one locus, it is quite evident that relatively more outcrossing is required to hold down homozygosity in the 2N population, a feature which at key loci is assumed traditionally to have deletereous effects on the bearer. The fly in this ointment

is the fact that, according to both Stebbins' (1976) views, and McPherson's observations (1972) on meiotic pairing in V. adunca, the 4N of this species is unlikely to be a strict autoploid, thus questioning the auto-4N model's assumption that we can freely play with combinations of four gene-copies at our hypothetical locus. The alternative alloploid model considers the 4N as a diploid, but includes two loci with non-interchangable alleles viz. a diploid two-locus model. The output in Appendix IV shows how this too differs from the diploid. In all events , it is quite likely that a diploid would benefit more from a sustained ability to outcross, than would the tetraploid, and it is therefore interesting to note the more easily triggered anthesis in the former. Whether or not there is a strategic link between the two facts could only really be considered with a much more comprehensive data-base.

Another interesting point with regard to selfing, but this time not related directly to ploidy, is the role of selfing in colonizing species as perceived by Allard (1965; 1975) His view, recognised as one of "tooth and claw" economy[10], is that selfing turns this assumed bugbear of homozygosity to the advantage of colonizing species by allowing close and rapid adaptive conformity to a diversity of habitat types. With a small amount of outcrossing, there would also be no problem in maintaining a flexibility within the genome, which might allow subsequent rapid adaptation to

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[10] Personal comment by C.Strobeck, Dept. of Genetics, U. of A.

other situations which might arise. Jain (1976) in his review article, regards the attainment of an optimum balance between selfing and outcrossing to be a potentially significant aspect of any propagational strategy involving these two modes of reproduction. At the same time he negates the idea that inbreeding as such affords a unique colonizing strategy. With the little information which we have regarding the genetics of V. adunca, it is difficult to know whether these thoughts are applicable to the apparently more successfully self-fertilizing 4N in its envisaged colonization of the post-Pleistocene till.

In summarizing this matter of outcrossing it is tempting to conclude that its advantages, made available to the 2N by more liberal flowering, allow for the adaptive fine tuning of a species to an already well established niche space, while inbreeding favoured the coarse and rapid adaptive characteristics of a more opportunistic colonizer. Coupled with Stebbins' (1974) remarks that "self-fertilizing species are usually the ends of evolutionary lines, and rarely, if ever, contribute to major evolutionary trends", an image of the gradual replacement of the 4N by the 2N over geological time is is easily generated. Gould (1977), in a popular treatise on evolutionary thought, points out that natural selection, divested of its (Victorian) bias of "progress", can be viewed solely as a tracking of environmental events. Could "tetraploidness" in V. adunca be regarded as merely a manifestation of the species under

particular extreme conditions?

One further point with regard to the relative merits of chasmogamous and cleistogamous flowers, is that of the energetics of flowering. According to Schemske (1978) in a study of Impatiens, cleistogamous flowers are one-half to one-third as costly on a per seed basis as their chasmogamous counterparts. This as applied to Viola, would account for an extra handicap to the 2N in any imagined race across the glacial deposits, should it be more reliant on outcrossing for effective propagation.

The other apparent propagational attribute of the diploid viz. the greater tendency to form plantlets from overwintered roots, can also be viewed as entrenchmennt behaviour. Formation of plantlets in the immediate vicinity of the previous years' parent plant (which, as we have seen will survive milder winters intact), would assure an individual or its cloned offspring of continued, or even enhanced presence at that point in space. This stategy would under the more extreme winters of the north, and in the absence of competition from the more rapidly regenerating 2N (see Fig. 20), also be available to the 4N in its competition with other species.

Although far from complete as a model, the above set of ideas may be composed to form a loose framework for an understanding of some of the possible mechanisms contributing to what we see as the distributions of the chromosome races of V. adunca. What these present-day

distributions are, of course, is a scenario intersected on the time axis at the present, and any attempt to explain them involves speculative projections back along that axis toward the Pleistocene. As has been pointed out, a lot has had to be assumed in order to take those journeys through time to the nunatak island of the Cypress Hills accomodating castaway diploid and (newly formed?) tetraploid populations of V. adunca, amidst a vast ocean of ice. In all likelihood this image is totally incorrect, especially with regard to the nature of the glacial activity (Ives et al., 1975).

In order to proceed any further with the understanding of the problem, a choice of emphasis would have to be made, involving one of two general directions. Either an analytic approach could be a adopted in which the assumptions about our m fixed dimensions would be investigated and tightened (and possibly the number of variable dimensions reduced even further to the supposed critical ones), or an attempt could be made to construct a picture as whole as possible by scrutinizing as many of the total n dimensions as are available.

In the first case, a possibility would be to attempt a reconstruction of the situation at the supposed post-Pleistocene time when the 2N and 4N were first faced with their physiological uniquenesses in their common environment. This could be achieved by the synthetic production of a 4N from 2N stock. These two artificial races could then be subjected to experimental investigation in the

certain knowledge that the two hypervolumes had not been distorted with respect to one another by ecotypic divergence over the intervening millennia. The major problem here is the fact that we would then be working with an autoploid, contrary to the indications of McPherson (1972) and Stebbins (1976) mentioned previously. Other hurdles would include the expected difficulty in obtaining tetraploids with a fitness comparable to that found in nature.

The other route of attempting to characterize as nearly as possible the essential niche hypervolumes of the two ploidy levels, and then comparing the two constructs, could well be embodied in extensive autecological studies. This approach, amenable to the itinerant ecologist, well prepared with time, assistance and equipment, is also frought with difficulty, and with no guarantee of ultimate success. The final scene, once achieved, may well show the perplexed experimenter attempting to compare two n-dimensional uncertainty clouds with no definite shape, and only abstract existence.

Undoubtedly the route to understanding will reveal itself with patience and a dedication to open-mindedness, and will consist of many compromises between the predetermined approaches of currently accepted sub-disciplines. This study, although obviously limited in scope, assists in expanding the data-base for the (open-ended?) investigation of polyploidy as an evolutionary mechanism.

Summary

A synopsis of the relative performances of the two chromosome races of Viola adunca is given below.

1. Cold adaptedness:

Generally no differences were observed between chromosome races in the cold hardiness of leaf tissue, seeds, seedlings and mature whole plants. Exceptions to this were:

1. Unhardened 14 day-old seedlings subjected to a stress temperature of -6C gave 45% and 0% (zero) mortality in 2N and 4N populations respectively (page 54);
2. In unhardened mature whole plants, 2N's were marginally more successful than 4N's in surviving a cold stress of -5C (page 56).

2. Productivity:

Tetraploids were more productive than diploids in the production of seeds (pages 61 and 62), and also in the ability of seedlings to increase their biomass (page 72). The seedling populations also had significantly different root:shoot ratios (4N>2N) at 50 and 57 days (page 72), and these ratios showed a marked decrease over an increasing temperature gradient (page 75). Germinability of 4N seeds was also significantly higher than that for 2N seeds (page 68).

3. Nitrogen-fixation:

Although no difference was shown between races in their ability to establish associations with nitrgen-fixing microorganisms, the existence of such associations was confirmed in many cases (pages 78 and 79).

Epilogue

The role of polyploidy as an adaptive and evolutionary mechanism once more appears to be confirmed by this study. But the details of its manifestation seem to be as unique as Viola adunca itself when compared to similar studies with other higher plant species, defying the notion that polyploidy might offer any particular type of adaptive advantage common to all species in which it occurs. This conclusion is not suprising when we consider the degree of disruption which must be invoked with a change as great as the doubling of a genome. The finely balanced interaction of regulatory and structural genes, and the incomprehensibly complex network of metabolic pathways can only be upset by an event such as this, and any successful equilibrium subsequent to the catastrophic event should not be expected to be anything but random in its expression.

In considering the spectrum between strict alloploidy and strict autoploidy, it remains debatable whether or not there exists a relationship between the degree of hybridization and the likelihood that a polyploid line will evolve along a path divergent from that of its diploid ancestor. In other words, it remains moot as to whether or not tetraploid Viola adunca truly represents Darwinian speciation as is implicit in Löve and Löves' (1975) assertion that it is deserving of full species status.

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VI. APPENDICES

In order to minimize burdening the main text with unnecessarily detailed information, much has been held for presentation in this chapter.

A. Appendix I.

Determination of Ploidy Level.

During the initial stages of the project, most effort was expended on the determination of the racial categorization of the experimental plants by counting the chromosome number of the cells of mitotically active tissue. This meristematic tissue was obtained by depotting moderately drought-stressed plants, and excising the young root-tips which were usually plentiful at the interface between soil and pot. These root-tips were then processed according to the method of Tijo and Levan (1950), a procedure commonly employed by the students of taxonomy in this department (e.g. McPherson, 1972; Wolf, 1977; Elisens, 1978).

In this method, the excised roots are placed immediately into test-tubes containing a 0.002M 8-hydroxy-quinoline solution, and fixed for two-and-a-half to three hours at 15C ± 2C, usually in any available CEC at that temperature. Following the fixing, and a thorough wash in distilled water, the samples are stained in a solution of 9 parts aceto-orcein stain to 1 part 1M HCl, where the stain consisted of 0.22g orcein crystals (Fisher) dissolved in 10ml glacial acetic acid, diluted with 12ml distilled water (Peacock, 1966). Staining is improved by gently heating with a bunsen flame of the watchglasses containing the stain and tissue. Care has to be taken not to apply too much heat, which denatures the nucleoproteins, forming little balls

from the chromatids and making a determination of chromosome number impossible.

Root-tips are then placed on a glass microscope slide, one or two per slide, in a drop of 45% acetic acid. All excess tissue is then removed, leaving as nearly as possible only undifferentiated tissue behind. A glass cover-slip is carefully placed over the sample, and the tissue squashed by inverting the slide and pressing down forcefully with one's thumb onto a paper towel. The edge is then sealed, in this case with a mixture of Canada balsam and paraffin wax kept molten over a low heat.

Having followed the above procedure, the the root-tip preparations of Viola adunca were inspected at 900X magnification (under oil immersion) on a Leitz Wetzlar compound microscope equipped with a green filter to enhance the image of the red stained chromatids. The plant from which any particular sample was taken was then categorized after two distinct counts had been made on two separate root-tips, a count of 20 assigning it to the diploid population, and 40 to the tetraploid. Triploids (30) were set aside and were not used in any of the subsequent experiments.

B. Appendix II.

Controlled environment facilities.

During this study much use was made of the extensive controlled environment facilities (CEF) housed by the Botany Department in the Biological Sciences Building on the U.of A. campus. The facilities, their specifications, and the regimes programmed are detailed below (or referenced elsewhere).

Step-in CEC

Manufacturer: Environmental Growth Chambers, Chagrin Falls, Ohio.

Model: M7.

Lighting: Full lighting was provided by 24 cool white fluorescent tubes and 10 incandescent lamps (100W each), and reduced lighting ("two-thirds") by 16 and 6 fluorescent and incandescent lamps respectively. (Details of the lighting regimes are given in Materials and Methods, and Results. Quantum flux values were measured with a Lamda LI185 meter with a matching quantum sensor). The light-cap in this chamber is separated from the interior by a plexiglass heat barrier, below which the suspended bed was set at approximately 0.8m.

Air circulation: Built-in fans moved the chamber air, always supplemented with make-up air from the exterior, at a speed of approximately 5m.s^{-1}. Deflectors were erected to shield

the plants from the full force of this.

Ambient and Dew-point Temperatures: When these were remotely controlled, they were done so by means of the Honeywell W806 cam-and-rider type controller, giving regimes described in Methods and Materials. Calibration of these two parameters was achieved by using a fine-wire thermocouple (0.125mm; copper-constantan; Omega Engineering, Connecticut) in conjunction with a digital read-out thermometer (Fluke, model 2100A), and a dew-point hygrometer of the condensing-mirror type (EG & G, model 880).

Reach-in CEC.

Manufacturer: As above.

Lighting: This cabinet was used solely for cold-stressing experiments, which were done in darkness.

Air movement: Air inside the chamber was circulated at a similar velocity to that in the step-in described above, producing air movement of approximately 1 to 2m.s^{-1} in the vicinity of the test material.

Ambient air temperature: When decreasing temperatures were required, they were controlled remotely with a spirally cut cam on the Honeywell cam-and-rider type controller (see Methods and Materials).

Dew-point temperature: No attempt was made to control or measure this, as saturation of the air was usually achieved during cooling.

Germination cabinet.

Manufacturer: Coldstream.

Interior size: 0.35m^3

Lighting: This was provided by two sets of 4 cool white fluorescent lamps arranged vertically down each side of the cabinet, and separated from the interior by glass barriers. Shelving within was arranged so as to give an approximate maximum of 75microEinsteins.$m^{-2}.s^{-1}$ from any direction at the position of the sample.

Ambient air temperature: The two-step controller was set to give a day/night regime of 20/10C ± 1C.

Humidity: Control of humidity via the LiCl-sensor and humidistat system enabled a high humidity (approximately 85%) to be maintained at all times.

Cold rooms.

These rooms, equipped with cooling compressors, control mechanisms and insulated siding provided by the same manufacturer as for the CEC's above, were kept dark, and at the constant temperatures described in Methods and Materials. Any material deposited in these rooms for any length of time was protected from desiccation by a polyethylene wrapping, thereby obviating the need to measure wind-speed or humidity.

Standard greenhouse.

This refers to any of the standard, double-glazed compartments of the Botany Department greenhouses. These are maintained at approximately 20C throughout the year, although air temperatures as high as 30C have been observed on particularly warm days in the spring before the lowering of the summer shades. These are slatted wooden shades painted silver and giving 50% shade.

Trop-arctic greenhouses

These are similar to the standard greenhouses in structure, but are equipped with heating and cooling apparati capable of attaining and maintaining a wider range of temperature regimes. This machinery includes a glycol heater and a brine cooling system, which regulate the air temperature in the closed loop. Humidification is achieved with a steam humidifier controlled by a LiCl sensor. Both temperature and humidity, like in the CEC's, are able to be remotely controlled by means of a cam-and-rider controller. Although supplementary lighting does exist, it was not used in this project, during which the trop-arctic greenhouses were only used in the summer months.

The four greenhouses used covered a range of temperature regimes, which could be represented along a conceptual gradient with units of measurement of degree(C) hours per day (see Results), which disguises the fact that

the one important parameter of periodicity was not standardized. The peculiarities of the individual regimes, which for practical reasons could not be altered, are described below.

3. Cold (134 degree hours per day).

   No thermoperiod, but five large defrost peaks per day with maximum values of 10 to 15C.

   Mean ambient air temperature: 5.6C

   Mean dew-point temperature: 0C

4. Cool (254 degree hours per day)

   No programmed thermoperiod, but influence of solar heating caused diurnal fluctuations in the range 7.5C to 13.0C.

   Mean ambient air temperature: 10.6C

   Mean dew-point temperature: 5.2C

5. Moderate (398 degree hours per day).

   Programmed to give 10h at approximately 12.5C, and 12h at approximately 19C, with a 2h transition before the warm period, while the dew-point temperature ranged from 10C to 12.5C.

   Mean ambient air temperature: 16.6C

   Mean dew-point temperature: 11.9C

6. Warm (540 degree hours per day).

   Programmed to give 4h at 20C, followed by a 6h

transition to 26C where it remained for 10h before returning to 20C in 4h.

Mean ambient air temperature: 22.7C

Mean dew-point temperature: 15.6C

C. Appendix III.

Site descriptions.

Site descriptons for the collection of sites of the plants used in the acetylene-reduction assay of rhizospheric nitrogenose activity. Approximate map references are given where available.

Trip of August, 1978.

a) East of Redwater on the verge of a secondary road, at the edge of a mixed stand of aspen, spruce and pine, amidst young aspen, rose, Arctostaphylos uva-ursi, Amelanchier, Vaccinium myrtilloides, Salix, various herbs and grasses. 53.9°N 113.0°W

b) North-east of Edmonton, 100m off highway 643 on a small south facing hill in an open stand of young aspen on fine sandy soil with Maianthenum and rose, as well as grasses. 53.8°N 113.2°W

c) Sterco, Alberta; on coalspoil site of the old Sterco Coal-mine; map reference 53.1°N 116.8°W; altitude 1408m.

d) Floodplain of the N. Saskatchewan River where the D. Thompson Highway crosses it near Rocky Mountain House, under Balsam poplar and White spruce, with Elaeagnus, Arctostaphylos, herbs and grasses. 52.4°N 114.9°W

e) On the west bank of the Big Horn Dam, 2.7 km

south of the Cline River settlement on the D. Thompson Highway, in a clearing in a Pinus contorta stand, amidst Arctosphylos, Linnaea borealis, juniper, Sheperdia, herbs and graminoids. 52.2°N 116.5°W

f) Opposite the entrance to the lumber yard at Canal Flats on Highway 93/95, in a stand of Pinus ponerosa with Amelanchier, Sheperdia, Elaeagnus, herbs and grasses. 50.2°N 115.8°W

g) West (2.7km) of Coleman on Highway 3, on a ridge North-East (downwind) of a sulpur plant site; previuosly seeded with Bromus and Medicago; relict native species include Sedum and Artemisia frigida. 49.6°N 114.5°W

h) Cranbrook, Jim Smith Lake campsite, on the crest of a ridge to the north east of the picnic site under Pinus contorta, Larix and Salix; other species include rose, juniper, Arctostaphylos, Fragaria and grasses. 49.5°N 115.8°W

j) Lundbreck, between Picher Creek and Bellevue, 0.6km up Rock Creek Rd., in patch of Elaeagnus, with Achillea and Bromus. 49.6°N 114.3°W

k) Woolford Prov. Park along the bank of the St. Mary's River under Salix and Balsam poplar, with Elaeagnus, Astragalus and Thermopsis. 49.2°N 113.1°W

m) South-west (approx. 10km) of Darby, Montana, along the Tin Cup Rd., in a very shaded site under Abies and Pinus ponderosa.

n) Denver, Colorado. (subspecies bellidifolia

(Greene) Harrington), near top of Guinella Pass to west of Georgetown at 11,165ft., on southeast-facing slope (approx. 40°), soil saturated by melting snowbank 200ft. above.

Trip of June, 1979.

In addition to revisting some of the above sites, the following were included:

a)East of the Reesor Lake in the Cypress Hills, on a shady site beneath aspen, growing in decaying leaf litter. 49.7°N 110.1°W

b)On a bench area to the north east of Graburn Monument in the Cypress Hills, adjacent to pine, but amongst Arctostaphylos, Linnaea, Sheperdia, legumes and grasses. 49.7°N 110.0°W

c) Opposite Lodgepole campsite in Cypress Hills alongside the main road amongst Arctostaphylos and grasses. 49.7°N 110.3°W

d) Between Fernie and Cranbrook, 4.3km east of Galloway, under P. contorta with Arctostaphylos. 49.3°N 115.2°W

e) Arctostophylos sample from the Kananaskis Research Centre near Seebe, Alberta. 51.0°N 115.0°W

D. Appendix IV.

Population genetics.

On the following pages are listed the Fortran programs which were written to simulate varying degrees of outcrossing in otherwise selfing populations of different ploidy levels. (An outcrossing labelled as 50% would mean that all individuals first propagate a replacement population by selfing, and then 50% of that progeny outcross and again add a replacement value to the total. That procedure constitutes one generation). In the data sets labelled as "OUTCROSSING ONLY", no selfing occurs.

The output from each program is listed after the program listing, and gives the population composition for progressive generations when starting with 100% heterozygosity. (In the case of the autoploid, this means two gene-copies bearing one allele, and the other two bearing another. In the alloploid it is taken to mean heterozygous at both of the two independent loci).

These simple models represent a very meagre skeleton, and ought really to be exploited more fully by introducing assumed advantage-weightings for different gene-combinations. Even without an empirical set of observed data, further manipulation might show up something of the nature of the maintenance of variability in 4N populations under conditions of selfing. A lack of time and easily mobilized modelling skill has prevented any expansion of these ideas at this time by the author.

```
C  PROGRAM "DIPLOID", TO CHECK THE DEGREE OF
C  HOMOZYGOSITY IN A PARTIALLY OUTCROSSING
C  POPULATION OF DIPLOIDS AFTER IM GENERATIONS.
       IOUT=0
       FOUT=0.
       ASSIGN 51 TO ISTAT
       GO TO 60
 51    FOUT=.1
       ASSIGN 52 TO ISTAT
       GO TO 60
 52    FOUT=0.5
       ASSIGN 53 TO ISTAT
       GO TO 60
 53    FOUT=1.0
       ASSIGN 54 TO ISTAT
       GO TO 60
 54    FOUT=1.1
       ASSIGN 55 TO ISTAT
 60    CONTINUE
       IOUT=IOUT+1
       IN=0
       FPC=FOUT*100.
       WRITE(6,77)
 77    FORMAT('1','DIPLOID')
       IF(100. - FPC)59,56,56
 56    CONTINUE
       WRITE(6,29)FPC
        GO TO 57
 59    WRITE(6,66)
 66    FORMAT('0','%AGE OF POPULATION
    * OUTCROSSING: OUTCROSSING ONLY.')


 57    CONTINUE
       WRITE(6,22)
 29    FORMAT('0','%AGE OF POPULATION OUTCROSSING:',F5.1)
 22    FORMAT('0',22X,'HOM',9X,'HET      ')
       HOM=0.
       HET=100.
       XPOP=0.
       IM=14
       WRITE(6,32)IN,HOM,HET
 7     IN=IN+1
C  COMPUTE PROGENY GENERATED BY SELFING,
C  CHOOSING TO SKIP FOR OUTXING BY SETTING
C  FOUT .GT. 1.0
        IF (FOUT .GT. 1.0)GOTO 11
C  SELFING OF DIPLOIDS BY PANMIXIS
C  OF 4 GAMETES PER INDIVIDUAL.
      HOMT=HOM + (2./6.)*HET
      HETT=(4./6.)*HET
      GO TO 19
```

```
 11     CONTINUE
        HOMT=HOM
        HETT=HET
        GO TO 20
 19     CONTINUE
C  ASSUMING THAT THE GENE FREQUENCY REMAINS
C  CONSTANT (50:50 FOR THE TWO ALLELES),
C  OUTXING WILL ALWAYS CONTRIBUTE TO THE
C  POPULATION IN A 1:2:1 RATIO VIZ. 1/2 HOM
C  PLUS 1/2 HET.
C  THEREFORE WE ADD ON THE CONTRIBUTION OF
C  THE OUTXERS, SETTING THEIR REPRODUCTIVE
C  CAPACITY AT REPLACEMENT.
        XPOP=FOUT*(HOMT + HETT)
        HOMT=HOMT + 0.5*XPOP
        HETT=HETT + 0.5*XPOP
        GO TO 23
C   ..AND FOR OUTXING ONLY....
 20     CONTINUE
        HOMT=0.5*(HOMT + HETT)
        HETT=HOMT
C  VIZ. INSTANTANEOUS EQUILIBRIUM AT 50:50
 23     CONTINUE
        Z=(HOMT + HETT)/100.
        HOMT=HOMT/Z
        HETT=HETT/Z
        HOM=HOMT
        HET=HETT
        WRITE(6,32)IN,HOMT,HETT
32      FORMAT(' GENERATION:',I2,3F12.1)
        IF(IN .LE. IM)GO TO 7
        GO TO ISTAT, (51, 52, 53, 54, 55)
55      CONTINUE
        STOP
        END
```

DIPLOID

%AGE OF POPULATION OUTCROSSING: 0.0

	HOM	HET
GENERATION: 0	0.0	100.0
GENERATION: 1	33.3	66.7
GENERATION: 2	55.6	44.4
GENERATION: 3	70.4	29.6
GENERATION: 4	80.2	19.8
GENERATION: 5	86.8	13.2
GENERATION: 6	91.2	8.8
GENERATION: 7	94.1	5.9
GENERATION: 8	96.1	3.9
GENERATION: 9	97.4	2.6
GENERATION:10	98.3	1.7
GENERATION:11	98.8	1.2
GENERATION:12	99.2	0.8
GENERATION:13	99.5	0.5
GENERATION:14	99.7	0.3
GENERATION:15	99.8	0.2

DIPLOID

%AGE OF POPULATION OUTCROSSING: 10.0

	HOM	HET
GENERATION: 0	0.0	100.0
GENERATION: 1	34.8	65.2
GENERATION: 2	56.0	44.0
GENERATION: 3	68.8	31.2
GENERATION: 4	76.5	23.5
GENERATION: 5	81.2	18.8
GENERATION: 6	84.1	15.9
GENERATION: 7	85.8	14.2
GENERATION: 8	86.9	13.1
GENERATION: 9	87.5	12.5
GENERATION:10	87.9	12.1
GENERATION:11	88.1	11.9
GENERATION:12	88.2	11.8
GENERATION:13	88.3	11.7
GENERATION:14	88.4	11.6
GENERATION:15	88.4	11.6

DIPLOID

%AGE OF POPULATION OUTCROSSING: 50.0

	HOM	HET
GENERATION: 0	0.0	100.0
GENERATION: 1	38.9	61.1
GENERATION: 2	56.2	43.8
GENERATION: 3	63.9	36.1
GENERATION: 4	67.3	32.7
GENERATION: 5	68.8	31.2
GENERATION: 6	69.5	30.5
GENERATION: 7	69.8	30.2
GENERATION: 8	69.9	30.1
GENERATION: 9	70.0	30.0
GENERATION:10	70.0	30.0
GENERATION:11	70.0	30.0
GENERATION:12	70.0	30.0
GENERATION:13	70.0	30.0
GENERATION:14	70.0	30.0
GENERATION:15	70.0	30.0

DIPLOID

%AGE OF POPULATION OUTCROSSING: 100.0

	HOM	HET
GENERATION: 0	0.0	100.0
GENERATION: 1	41.7	58.3
GENERATION: 2	55.6	44.4
GENERATION: 3	60.2	39.8
GENERATION: 4	61.7	38.3
GENERATION: 5	62.2	37.8
GENERATION: 6	62.4	37.6
GENERATION: 7	62.5	37.5
GENERATION: 8	62.5	37.5
GENERATION: 9	62.5	37.5
GENERATION: 10	62.5	37.5
GENERATION: 11	62.5	37.5
GENERATION: 12	62.5	37.5
GENERATION: 13	62.5	37.5
GENERATION: 14	62.5	37.5
GENERATION: 15	62.5	37.5

DIPLOID

%AGE OF POPULATION OUTCROSSING: OUTCROSSING ONLY.

	HOM	HET
GENERATION: 0	0.0	100.0
GENERATION: 1	50.0	50.0
GENERATION: 2	50.0	50.0
GENERATION: 3	50.0	50.0
GENERATION: 4	50.0	50.0
GENERATION: 5	50.0	50.0
GENERATION: 6	50.0	50.0
GENERATION: 7	50.0	50.0
GENERATION: 8	50.0	50.0
GENERATION: 9	50.0	50.0
GENERATION:10	50.0	50.0
GENERATION:11	50.0	50.0
GENERATION:12	50.0	50.0
GENERATION:13	50.0	50.0
GENERATION:14	50.0	50.0
GENERATION:15	50.0	50.0

```
C  PROGRAM "AUTOPLOID", DESIGNED TO COMPUTE THE
C  DEGREE OF HOMOZYGOSITY IN A POPULATION OF
C  PARTIALLY OUTCROSSING AUTOTETRAPLOIDS,
C  AFTER IM GENERATIONS.
       GO TO 99
 97    CONTINUE
      IN=0
       FPC=FOUT*100.
       WRITE(6,77)
 77    FORMAT('1','AUTOPLOID')
       IF(100. - FPC)59,56,56
 59    WRITE(6,66)
 66    FORMAT('0','%AGE OF POPULATION
    *OUTCROSSING: OUTCROSSING ONLY')
       GO TO 57
 56    CONTINUE
       WRITE(6,36)FPC
 36    FORMAT('0','%AGE OF POPULATION OUTCROSSING:',F5.1)
 57    CONTINUE
       WRITE(6,33)
 33    FORMAT('0',23X,'HOMO',6X,'HET31',6X,'HET22')
       HOM=0.
       HET31=0.
       HET22=100.
       IM=14
       WRITE(6,32)IN,HOM,HET31,HET22
  7    IN=IN+1
C  COMPUTE GENOTYPES AFTER SELFING,
C  BUT IN ORDER TO ALLOW FOR OUTXING
C  ONLY, SET FOUT .GT. 1.
       IF (FOUT .GT. 1.) GO TO 11
       HOMT=36.*HOM + 18.*HET31 + 2.*HET22
        HET31T=16.*HET22
       HET22T=18.*HET31 + 18.*HET22
       TOT=(HOMT + HET31T + HET22T)/100.
       HOMT=HOMT/TOT
       HET31T=HET31T/TOT
       HET22T=HET22T/TOT
       GO TO 19
 11    CONTINUE
       HOMT=HOM
       HET31T=HET31
       HET22T=HET22
 19    CONTINUE
C  COMPTUE % OF POPULATION THAT WE WOULD
C  LIKE TO HAVE OUTCROSS (FOUT IS THE FACTOR).
C COMPUTE THE FREQUENCY OF GAMETES GENERATED BY
C  THE DIFFERENT GENOTYPES, 6 PER INDIVIDUAL FOR THE
C  OUTXING
C  PORTION OF THE POPULATION. TWO TYPES OF GAMETES,
```

```
C  THE HOMOZYGOUS TYPE BEING DIVIDED SYMMETRICALLY
C  FOR THE TWO ALLELES.
       HOMGAM=(6.*HOMT + 3.*HET31T + 2.*HET22T)
       HOMGAM=HOMGAM/2.
C  (....THAT WAS FOR THE TWO ALLELIC TYPES).
       HETGAM=(3.*HET31T + 4.*HET22T)
C  NOW COMPUTE THE FINAL PROGENIES, ACCOUNTING
C  FOR BOTH SELFING AND OUTXING.
      HOMX=2.*(HOMGAM**2)
       HET31X=4.*(HOMGAM*HETGAM)
       HET22X=HETGAM**2 + 2.*(HOMGAM**2)
       TOTX=(HOMX+HET31X+HET22X)/100.
       IF(FOUT .GT. 1.)GOTO 21
       HOMT=HOMT + (HOMX/TOTX)*FOUT
       HET31T=HET31T + (HET31X/TOTX)*FOUT
       HET22T=HET22T + (HET22X/TOTX)*FOUT
       GO TO 22
 21    HOMT=HOMX/TOTX
       HET31T=HET31X/TOTX
       HET22T=HET22X/TOTX
 22    CONTINUE
       Z=(HOMT+HET31T+HET22T)/100.
       HOMT=HOMT/Z
       HET31T=HET31T/Z
       HET22T=HET22T/Z
       HOM=HOMT
       HET31=HET31T
       HET22=HET22T
       WRITE(6,32)IN,HOMT,HET31T,HET22T
32     FORMAT(' GENERATION:',I3,3F12.1)
       IF(IN .LE. IM)GO TO 7
       GO TO ISTAT, (51, 52, 53, 54, 55)
99     CONTINUE
       IOUT=0
       FOUT=0.
       ASSIGN 51 TO ISTAT
       GO TO 60
51     FOUT=.1
       ASSIGN 52 TO ISTAT
       GO TO 60
52     FOUT=0.5
       ASSIGN 53 TO ISTAT
       GO TO 60
53     FOUT=1.0
       ASSIGN 54 TO ISTAT
       GO TO 60
54     FOUT=1.1
       ASSIGN 55 TO ISTAT
60     CONTINUE
       IOUT=IOUT+1
98     GO TO 97
55     CONTINUE
       STOP
       END
```

AUTOPLOID

%AGE OF POPULATION OUTCROSSING: 0.0

	HOMO	HET31	HET22
GENERATION: 0	0.0	0.0	100.0
GENERATION: 1	5.6	44.4	50.0
GENERATION: 2	30.6	22.2	47.2
GENERATION: 3	44.3	21.0	34.7
GENERATION: 4	56.7	15.4	27.9
GENERATION: 5	66.0	12.4	21.6
GENERATION: 6	73.4	9.6	17.0
GENERATION: 7	79.1	7.6	13.3
GENERATION: 8	83.6	5.9	10.4
GENERATION: 9	87.2	4.6	8.2
GENERATION: 10	90.0	3.6	6.4
GENERATION: 11	92.1	2.8	5.0
GENERATION: 12	93.8	2.2	3.9
GENERATION: 13	95.2	1.7	3.1
GENERATION: 14	96.2	1.4	2.4
GENERATION: 15	97.0	1.1	1.9

AUTOPLOID

%AGE OF POPULATION OUTCROSSING: 10.0

	HOMO	HET31	HET22
GENERATION: 0	0.0	0.0	100.0
GENERATION: 1	5.9	44.9	49.2
GENERATION: 2	29.8	24.3	45.9
GENERATION: 3	42.5	22.6	34.9
GENERATION: 4	53.1	17.7	29.2
GENERATION: 5	60.5	14.9	24.5
GENERATION: 6	66.1	12.7	21.3
GENERATION: 7	70.1	11.0	18.9
GENERATION: 8	73.0	9.8	17.1
GENERATION: 9	75.2	8.9	15.9
GENERATION: 10	76.8	8.3	14.9
GENERATION: 11	77.9	7.8	14.3
GENERATION: 12	78.7	7.5	13.8
GENERATION: 13	79.3	7.2	13.4
GENERATION: 14	79.8	7.0	13.2
GENERATION: 15	80.1	6.9	13.0

AUTOPLOID

%AGE OF POPULATION OUTCROSSING: 50.0

	HOMO	HET31	HET22
GENERATION: 0	0.0	0.0	100.0
GENERATION: 1	7.0	46.1	46.9
GENERATION: 2	27.5	30.1	42.4
GENERATION: 3	37.3	27.4	35.3
GENERATION: 4	43.8	24.1	32.1
GENERATION: 5	47.6	22.3	30.1
GENERATION: 6	49.8	21.2	29.0
GENERATION: 7	51.2	20.5	28.3
GENERATION: 8	52.0	20.1	28.0
GENERATION: 9	52.5	19.8	27.7
GENERATION: 10	52.7	19.7	27.6
GENERATION: 11	52.9	19.6	27.5
GENERATION: 12	53.0	19.5	27.5
GENERATION: 13	53.1	19.5	27.4
GENERATION: 14	53.1	19.5	27.4
GENERATION: 15	53.1	19.5	27.4

AUTOPLOID

%AGE OF POPULATION OUTCROSSING: 100.0

	HOMO	HET31	HET22
GENERATION: 0	0.0	0.0	100.0
GENERATION: 1	7.7	46.9	45.4
GENERATION: 2	25.6	34.2	40.2
GENERATION: 3	33.5	31.3	35.3
GENERATION: 4	37.8	28.8	33.4
GENERATION: 5	39.9	27.7	32.4
GENERATION: 6	41.0	27.0	32.0
GENERATION: 7	41.6	26.7	31.7
GENERATION: 8	41.8	26.5	31.6
GENERATION: 9	42.0	26.5	31.6
GENERATION: 10	42.0	26.4	31.5
GENERATION: 11	42.1	26.4	31.5
GENERATION: 12	42.1	26.4	31.5
GENERATION: 13	42.1	26.4	31.5
GENERATION: 14	42.1	26.4	31.5
GENERATION: 15	42.1	26.4	31.5

AUTOPLOID

%AGE OF POPULATION OUTCROSSING: OUTCROSSING ONLY

	HOMO	HET31	HET22
GENERATION: 0	0.0	0.0	100.0
GENERATION: 1	5.6	44.4	50.0
GENERATION: 2	9.9	49.4	40.7
GENERATION: 3	11.6	49.9	38.5
GENERATION: 4	12.2	50.0	37.8
GENERATION: 5	12.4	50.0	37.6
GENERATION: 6	12.5	50.0	37.5
GENERATION: 7	12.5	50.0	37.5
GENERATION: 8	12.5	50.0	37.5
GENERATION: 9	12.5	50.0	37.5
GENERATION: 10	12.5	50.0	37.5
GENERATION: 11	12.5	50.0	37.5
GENERATION: 12	12.5	50.0	37.5
GENERATION: 13	12.5	50.0	37.5
GENERATION: 14	12.5	50.0	37.5
GENERATION: 15	12.5	50.0	37.5

```
C  PROGRAM "ALLOPLOID" DESIGNED TO COMPUTE THE
C  DEGREE OF HOMOZYGOSITY IN A POPULATION OF
C  ALLOPOLYPLOIDS (=A DIPLOID TWO-LOCUS PROBLEM)
C  AFTER IM GENERATIONS OF PARTIAL OUTCROSSING.
      DIMENSION XPOP(3,3)
      GO TO 99
 97   CONTINUE
      IN=0
      FPC=FOUT*100.
      WRITE(6,77)
 77   FORMAT('1','ALLOPLOID')
      IF(100. - FPC)59,56,56
 59   WRITE(6,66)
      GO TO 57
 56   CONTINUE
      WRITE(6,36)FPC
 66   FORMAT('0','%AGE OF POPULATION
    *OUT CROSSING: OUTCROSSING ONLY')
 36   FORMAT('0','%AGE OF POPULATION OUTCROSSING:',F5.1)
 57   CONTINUE
      WRITE(6,33)
 33   FORMAT('0',18X,'HOMO      HET1      HET2')
      HOM=0.
      HET1=0.
      HET2=100.
      IM=14
      WRITE(6,32)IN,HOM,HET1,HET2
  7   IN=IN+1
C  COMPUTE GENOTYPES IN POPULATION AFTER ONE ROUND
C  OF SELFING, BUT IN ORDER TO ALLOW FOR COMPLETE OUTXING
C  ONLY, SET FOUT .GT. 1.0
      IF (FOUT .GT. 1.0)GO TO 11
      HOMT=16.*HOM + 8.*HET1 + 4.*HET2
      HET1T=8.*HET1 + 8.*HET2
      HET2T=4.*HET2
      GO TO 19
 11   CONTINUE
      HOMT=HOM
C  WHAT ARE THE RELATIVE FREQUENCIES OF
C  THE GENOTYPES STORED IN THE MATRIX XPOP?
C  THE ROWS OF THIS MATRIX ARE:
C              AABB   AABY   AAYY
C              AXBB   AXBY   AXYY
C              XXBB   XXBY   XXYY
C       WHERE X IS AN ALLELE OF A,
C         AND Y IS AN ALLELE OF B.
      HET1T=HET1
      HET2T=HET2
 19   CONTINUE
      TOT=(HOMT + HET1T + HET2T)/100.
```

```
      HOMT=HOMT/TOT
      HET1T=HET1T/TOT
      HET2T=HET2T/TOT
C  HOMOZYGOUS
      XPOP(1,1)=HOMT/4.
      XPOP(3,1)=XPOP(1,1)
      XPOP(1,3)=XPOP(1,1)
      XPOP(3,3)=XPOP(1,1)
C  HETEROZYGOUS AT 1 LOCUS.
      XPOP(1,2)=HET1T/4.
      XPOP(2,1)=XPOP(1,2)
      XPOP(2,3)=XPOP(1,2)
      XPOP(3,2)=XPOP(1,2)
C  AND LASTLY, HETEROZYGOUS AT 2 LOCI.
      XPOP(2,2)=HET2T
C  NOW COMPUTE THE FREQUENCY OF GAMETE TYPES
C  WHERE X AND Y REPRESENT THE ALTERNATIVE
C  ALLELES AT LOCI A AND B RESPECTIVELY.
      AB=4.*XPOP(1,1) + 2.*XPOP(1,2) + 2.*XPOP(2,1) +
     * XPOP(2,2)
      AY= 2.*XPOP(1,2) +4.*XPOP(1,3) + XPOP(2,2) +
     * 2.*XPOP(2,3)
      XB=2.*XPOP(2,1) + XPOP(2,2) +4.*XPOP(3,1) +
     * 2.*XPOP(3,2)
      XY=XPOP(2,2) + 2.*XPOP(2,3) + 2.*XPOP(3,2) +
     * 4.*XPOP(3,3)
C  ....AND THE FREQUENCY OF THE GENOTYPES THAT FOLLOWS....
      HOMX=AB**2 + AY**2 + XB**2 + XY**2
      HET1X=2.*(XY*XB + XY*AY + AB*XB + AB*AY)
      HET2X=2.*(AB*XY + AY*XB)
      TOTX=(HOMX + HET1X + HET2X)/100.
      IF (FOUT .GT. 1.)GO TO 21
      HOMT=HOMT + (HOMX/TOTX)*FOUT
      HET1T=HET1T + (HET1X/TOTX)*FOUT
      HET2T=HET2T + (HET2X/TOTX)*FOUT
      GO TO 22
 21   CONTINUE
      HOMT=HOMX/TOTX
      HET1T=HET1X/TOTX
      HET2T=HET2X/TOTX
 22   CONTINUE
C  AND THAT SHOULD DO IT
C  ARITHMETIC HAS BEEN LEFT UNABBREVIATED TO ALLOW
C  FOR EASIER CHECKING.
      Z=(HOMT+HET1T+HET2T)/100.
      HOMT=HOMT/Z
      HET1T=HET1T/Z
      HET2T=HET2T/Z
      HOM=HOMT
      HET1=HET1T
      HET2=HET2T
      WRITE(6,32)IN,HOMT,HET1T,HET2T
 32      FORMAT(' GENERATION:',I2,3F10.3)
      IF(IN .LE. IM)GO TO 7
```

```
      GO TO ISTAT, (51, 52, 53, 54, 55)
99    CONTINUE
      IOUT=0
      FOUT=0.
      ASSIGN 51 TO ISTAT
      GO TO 60
51    FOUT=.1
      ASSIGN 52 TO ISTAT
      GO TO 60
52    FOUT=0.5
      ASSIGN 53 TO ISTAT
      GO TO 60
53    FOUT=1.0
      ASSIGN 54 TO ISTAT
      GO TO 60
54    FOUT=1.1
      ASSIGN 55 TO ISTAT
60    CONTINUE
      IOUT=IOUT+1
98    GO TO 97
55    CONTINUE
      STOP
      END
```

ALLOPLOID

%AGE OF POPULATION OUTCROSSING: 0.0

	HOMO	HET1	HET2
GENERATION: 0	0.0	0.0	100.000
GENERATION: 1	25.000	50.000	25.000
GENERATION: 2	56.250	37.500	6.250
GENERATION: 3	76.563	21.875	1.563
GENERATION: 4	87.891	11.719	0.391
GENERATION: 5	93.848	6.055	0.098
GENERATION: 6	96.899	3.076	0.024
GENERATION: 7	98.444	1.550	0.006
GENERATION: 8	99.220	0.778	0.002
GENERATION: 9	99.610	0.390	0.000
GENERATION:10	99.805	0.195	0.000
GENERATION:11	99.902	0.098	0.000
GENERATION:12	99.951	0.049	0.000
GENERATION:13	99.976	0.024	0.000
GENERATION:14	99.988	0.012	0.000
GENERATION:15	99.994	0.006	0.000

ALLOPLOID

%AGE OF POPULATION OUTCROSSING: 10.0

	HOMO	HET1	HET2
GENERATION: 0	0.0	0.0	100.000
GENERATION: 1	25.000	50.000	25.000
GENERATION: 2	53.409	38.636	7.955
GENERATION: 3	70.196	25.723	4.081
GENERATION: 4	78.707	18.093	3.200
GENERATION: 5	82.776	14.224	3.000
GENERATION: 6	84.671	12.375	2.955
GENERATION: 7	85.543	11.513	2.944
GENERATION: 8	85.941	11.117	2.942
GENERATION: 9	86.123	10.936	2.941
GENERATION:10	86.206	10.853	2.941
GENERATION:11	86.243	10.816	2.941
GENERATION:12	86.260	10.799	2.941
GENERATION:13	86.268	10.791	2.941
GENERATION:14	86.272	10.787	2.941
GENERATION:15	86.273	10.786	2.941

ALLOPLOID

%AGE OF POPULATION OUTCROSSING: 50.0

	HOMO	HET1	HET2
GENERATION: 0	0.0	0.0	100.000
GENERATION: 1	25.000	50.000	25.000
GENERATION: 2	45.833	41.667	12.500
GENERATION: 3	54.861	34.722	10.417
GENERATION: 4	58.218	31.713	10.069
GENERATION: 5	59.394	30.594	10.012
GENERATION: 6	59.796	30.202	10.002
GENERATION: 7	59.932	30.068	10.000
GENERATION: 8	59.977	30.023	10.000
GENERATION: 9	59.992	30.008	10.000
GENERATION:10	59.997	30.003	10.000
GENERATION:11	59.999	30.001	10.000
GENERATION:12	60.000	30.000	10.000
GENERATION:13	60.000	30.000	10.000
GENERATION:14	60.000	30.000	10.000
GENERATION:15	60.000	30.000	10.000

ALLOPLOID

%AGE OF POPULATION OUTCROSSING:100.0

	HOMO	HET1	HET2
GENERATION: 0	0.0	0.0	100.000
GENERATION: 1	25.000	50.000	25.000
GENERATION: 2	40.625	43.750	15.625
GENERATION: 3	45.703	39.844	14.453
GENERATION: 4	47.119	38.574	14.307
GENERATION: 5	47.491	38.220	14.288
GENERATION: 6	47.587	38.127	14.286
GENERATION: 7	47.611	38.103	14.286
GENERATION: 8	47.617	38.097	14.286
GENERATION: 9	47.619	38.096	14.286
GENERATION:10	47.619	38.095	14.286
GENERATION:11	47.619	38.095	14.286
GENERATION:12	47.619	38.095	14.286
GENERATION:13	47.619	38.095	14.286
GENERATION:14	47.619	38.095	14.286
GENERATION:15	47.619	38.095	14.286

ALLOPLOID

%AGE OF POPULATION OUTCROSSING: OUTCROSSING ONLY

	HOMO	HET1	HET2
GENERATION: 0	0.0	0.0	100.000
GENERATION: 1	25.000	50.000	25.000
GENERATION: 2	25.000	50.000	25.000
GENERATION: 3	25.000	50.000	25.000
GENERATION: 4	25.000	50.000	25.000
GENERATION: 5	25.000	50.000	25.000
GENERATION: 6	25.000	50.000	25.000
GENERATION: 7	25.000	50.000	25.000
GENERATION: 8	25.000	50.000	25.000
GENERATION: 9	25.000	50.000	25.000
GENERATION:10	25.000	50.000	25.000
GENERATION:11	25.000	50.000	25.000
GENERATION:12	25.000	50.000	25.000
GENERATION:13	25.000	50.000	25.000
GENERATION:14	25.000	50.000	25.000
GENERATION:15	25.000	50.000	25.000

$$\int_0^{2\times10^{-2}\,\text{Darwins}} f(T, \psi, LD_{50})\,dt \neq 0 \overset{?}{\Rightarrow} 2N \neq 4N$$

i want to be alone !