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A STUDY ON THE INDUCTION OF FLOWERING IN *LOLIUM MULTIFLORUM*

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A STUDY ON THE INDUCTION OF FLOWERING IN

LOLIUM MULTIFLORUM

by

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I devote this thesis to my parents,
Murray and Susan Landman.

ABSTRACT

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Lolium multiflorum Lam. is, as a pasture grass, the most widely used annual temperate grass species in the higher rainfall areas of South Africa, particularly so in the Natal Midlands, the Eastern Highveld, the Eastern Cape and the winter rainfall areas of the Western Cape. Under irrigation, the popularity of the species is rapidly increasing in areas other than those mentioned above. These pastures are expensive and used primarily for high-producing animals, such as dairy cows and fat lamb production.

Cultivars that are mixtures of Italian and Westerwolds type plants are, because of their production value, well adapted to South African conditions. Italian and Westerwolds type plants, however, have different requirements for the induction of flowering. Thus, correct management of seed production units consisting of mixed cultivars is of vital importance if genetic shift and reduced seed yield are to be avoided.

The aim of this study was to determine the vernalization requirements of seed and plants of some L. multiflorum cultivars, as well as the possible translocation of the vernalization stimulus. Different vernalization techniques were introduced and the use of biotechnological techniques was investigated.

It was concluded that seed production units containing Italian type plants should be planted in autumn, in order to be vernalized as plants in winter. These plants must go through a juvenile phase before becoming receptive to vernalization. Translocation of the vernalization stimulus did not occur in this study. It is recommended that the close down date for seed production units should be revised, if maximum seed yield, genetic stability and the composition of mixed cultivars are to be maintained.

'N ONDERSOEK NA BLOMINDUKSIE BY LOLIUM MULTIFLORUM

deur

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Lolium multiflorum Lam. word, as weidingsgras, die meeste van alle eenjarige gematigde grasspesies in die hoër reënvalgebiede van Suid-Afrika gebruik, veral in die Natalse middellande, die oostelike Hoëveld, die Oos-Kaap en die winterreënvalgebiede van die Wes-Kaap. Onder besproeiing neem die gewildheid van die spesie vinnig toe in ander gebiede as die reeds genoem. Hierdie aangeplante weidings is duur en word hoofsaaklik vir hoogs-produserende diere gebruik, soos byvoorbeeld melkkoeie en die afronding van lammers.

Cultivars wat uit mengsels van Italiaanse en Westerwolds tipes plante bestaan, is as gevolg van hul produksiewaarde goed aangepas vir Suid-Afrikaanse toestande. Hierdie twee tipes plante het egter verskillende vereistes vir blominduksie. Die korrekte bestuur van saadproduksie-eenhede bestaande uit gemengde cultivars is dus van die uiterste belang indien genetiese verskuiwing en 'n vermindering in saadproduksie voorkom wil word.

Die doel van hierdie studie was om die vernalisasievereistes van saad en plante van verskillende L. multiflorum cultivars asook die moontlike translokering van die vernalisasie-stimulus, vas te stel. Verskeie vernalisasietegnieke is gebruik en biotegnologiese tegnieke is ondersoek.

Daar is vasgestel dat saadproduksie-eenhede wat Italiaanse tipe plante bevat in die herfs geplant behoort te word, om te verseker dat dit in die winter as plante gevernaliseer word. Hierdie plante moet deur 'n jeugfase gaan voordat dit ontvanklik word vir vernalisasie. Translokering van die vernalisasiestimulus het nie in hierdie studie plaasgevind nie. Die afsnydatum vir saadproduksie-eenhede behoort hersien te word indien maksimum saadopbrengs, genetiese stabiliteit en die samestelling van gemengde cultivars gehandhaaf wil word.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND TERMINOLOGY

CLOSE DOWN DATE - the date determined by the Plant Improvement Act by which grazing animals must be withdrawn from seed production units.

LDP (long-day plant) - plants that only flower when the day length is longer than some critical minimum

OECD - Organisation for Economic Co-operation and Development

PHOTOPERIOD - daylength

SANSOR - South African National Seed Organisation

SDP (short-day plants) - plants that only flower when the day length is shorter than some critical maximum

SDS PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

TILLER - a branch produced from the base of a stem

CHAPTER 1

GENERAL INTRODUCTION, LITERATURE REVIEW AND MORPHOLOGICAL STUDY

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GENERAL INTRODUCTION, LITERATURE REVIEW AND MORPHOLOGICAL STUDY

1.1 General introduction

Lolium multiflorum Lam. (Italian ryegrass) is indigenous to the mediterranean parts of Europe and Northern Africa and to the temperate parts of Asia. At present it is cultivated over most of the temperate as well as many tropical parts of the world. As a pasture grass it is the most widely used annual temperate grass species in the higher rainfall areas of South Africa, particularly so in the Natal Midlands, the Eastern Highveld, the Eastern Cape and the winter rainfall areas of the Western Cape.

Under irrigation, the popularity of the species is rapidly increasing in areas other than those mentioned above, where it fulfils the need for high quality fodder in late autumn and especially in spring. Although not generally recommended, the grass may be grown under dryland conditions, provided that the rainfall exceeds 900mm per annum and that most of the precipitation occurs in autumn, winter and spring. As a result of the high moisture and nutrient requirements, these annual ryegrass pastures are expensive and consequently are used primarily for high-producing animals, such as dairy cows and fat lamb production.

SANSOR estimates that the market for annual ryegrass seed handled by the seed trade is 1,185 tons of certified seed per year, made up of 1 000 tons of Midmar and 185 tons of imported cultivars. Very substantial over-the-fence sales also take place and the estimated real seed sales exceed 2 000 tons.

Ryegrass cultivars which are commercially available in South Africa can be classified into three main categories, namely relatively pure Italian types, relatively pure Westerwolds types as well as mixtures of the two types. Midmar, by far the most widely used cultivar in South Africa, is a typical example of a mixture and was bred by selecting and crossing adapted plants from cultivars with both Italian and Westerwolds characteristics. After this

procedure, a rust tolerant cultivar, Midmar, was registered and inscribed on the South African Variety List.

Until 1976, ryegrass pastures in South Africa were based on imported seed and locally produced "common" cultivars of undescribed origin. With the release of Midmar in 1976 and the production of certified seed in South Africa, the ryegrass seed industry came to its own. The market share of Midmar grew rapidly. Subsequently the seed production of Midmar moved to areas which differ climatically from the Natal Midlands, where Midmar was developed. These areas vary from the Eastern Transvaal Highveld, Eastern Free State and Eastern Cape, with cold winters, to the central coastal area, where no frost occurs. After Midmar had been released, with the overseas evaluation of Midmar, it came, for the first time, to the breeders' attention that Midmar is not a true Italian cultivar, but includes 80% Westerwolds type plants. Only in the eighties when farmers started establishing pastures in spring, they noted that only some of the plants flowered (Westerwolds types) while the other plants (Italian types) went through eighteen months without flowering. Only then did they realise that Midmar, for seed production, should probably not be established in spring, but in autumn, so that it can be exposed to a sufficient cold period. As a mixture, Midmar is well adapted to South African conditions because of the production value of this "mixture".

In the South African context, where there is a shortage of fodder in late autumn, winter and spring, the dry matter production curves of true Italian and true Westerwolds cultivars do not conform to the wide spectrum of requirement. Westerwolds type plants produce better in winter, while Italian type plants are superior in spring and early summer. Thus, cultivars consisting of mixtures result in an improved fodder flow, as the production curves complement each other in mixtures.

Italian and Westerwolds ryegrass cultivars have different requirements for the induction of the reproductive phase. The Italian type plants require vernalization (a period of winter cold) followed by an increase in daylength and warmer temperatures for the initiation of flowering. However, the specific cold requirements for different cultivars, as well as for individual plants within cultivars may vary. It has also been noted that new tillers which

are formed after the winter cold has passed, often may not enter into the reproductive phase unless they become vernalized by unseasonal cold weather. In contrast to Italian types, Westerwolds type plants do not need vernalization for the induction of flowering. They flower in response to increasing daylength and/or rising temperatures.

If the Westerwolds and Italian types do have different requirements for the induction of flowering, a variation in production areas can cause genetic shift, especially in temperate areas. In these areas a mixed cultivar, such as Midmar, can become entirely Westerwolds. Further problems may occur on the Eastern Transvaal Highveld, where sheep are put onto ryegrass pastures until after 15 October. Vernalized (initiated) tillers may be grazed off and the Italian component of the seed decreases.

The differing requirements of Italian and Westerwolds plants for the induction of flowering have very important implications with respect to seed yields, the maintenance of genetic stability and composition and the Seed Certification Scheme. The legislated close-down date for extracting grazing animals from certified seed units of both Italian and Westerwolds cultivars is 15 October. In many seed-producing areas, closing down as late as 15 October may result in the removal of reproductive tillers. Westerwolds cultivars will form new tillers which will go into the reproductive phase and still have satisfactory seed yields. Italian cultivars, on the other hand will form tillers which, in the absence of a cold period will remain in the vegetative phase and result in a drastically reduced seed yield. Correct management of seed production units is of vital importance if genetic shift is to be avoided. This study is therefore important to establish management guidelines whereby the composition of the cultivar can be maintained.

The aim of this study was to determine the vernalization requirements of seed and plants of L. multiflorum cultivars traditionally used in the R.S.A., as well as the possible translocation of the vernalization stimulus. To achieve this aim, different vernalization techniques were introduced on imbibed seeds, container grown plants, and meristems grown in vitro. The use of electrophoresis to determine whether plants have been vernalized or not, was also investigated.

1.2 Literature review

Vernalization is defined as "the specific promotion of flowering by a cold treatment given to the imbibed seed or young plant" (Wilkins, 1984).

Systematic research on the subject was undertaken in 1857, by Klippart (Chouard, 1960). He showed that among the various climatic factors of winter, the determining factor is the cold temperature to which the young plants are subjected for a few weeks. This makes winter cereals capable of flowering soon after the onset of warmer temperatures. Subsequently Gassner showed that in winter cereals the fully imbibed seed is already sensitive to the specific cold effect, while, in 1928, Lysenko established that slight imbibition makes cereal seed "susceptible to this action of cold" (Chouard, 1960).

Additional support for the importance of temperature as an initiator of flowering came from the studies of Gassner in 1918 on the flowering of cultivated varieties of cereals. The technique of cold treatment came to be known as vernalization and was subsequently extended to exposure to cold not only at the seed stage, but also at later stages of development of the plant. Generally species which can be vernalized at the seed stage are facultative cold-requiring plants, while those which can only be vernalized at the plant stage have an obligate chilling requirement (Wareing & Phillips, 1978).

One of the most striking features of vernalization is that it appears to involve processes which proceed more rapidly at low than at higher temperatures. Such an effect is most unusual for chemical processes and one must assume that the changes occurring during vernalization are essentially enzyme-controlled reactions showing the usual characteristics of such reactions (Wareing & Phillips, 1978).

Temperature has a direct effect on floral initiation in some plants, but this can be distinguished from vernalization, as the latter is an inductive phenomenon. Floral initials are not yet present once vernalization has occurred, but they differentiate later, once the plant is returned to higher temperatures and, in many cases, to particular photoperiodic regimes. De-vernalization can occur at high temperatures or short photoperiods (Wilkins,

1984).

For the majority of species, the most effective temperatures for vernalization are just above freezing, but temperatures ranging from -1°C to 9°C are almost equally effective. Freezing of the cells is not necessary, suggesting that physiological processes are involved (Wareing & Phillips, 1978).

Chilling is only effective when it is applied over several days, weeks or months in the presence of oxygen to moist tissues containing enough carbohydrate to support adequate respiration. Concomitant growth may be moderate or slow. If growth stops completely, vernalization does not occur (Chouard, 1960). Vernalization is perceived by bud primary meristems and is sensed at a certain age which may be very early (immature embryo), or later (leafy plant), depending on the species (Chouard, 1960).

In L. multiflorum it was found that reproductive development occurred sooner in plants grown at lower temperatures and that it did not occur in those grown at day/night temperatures of 24/19 and 30/25°C respectively (Hill & Pearson, 1985). Knowing that Italian type plants require vernalization for the induction of flowering, it can be assumed that Hill & Pearson worked with pure Italian types.

A vernalization requirement for flowering is often linked with a particular photoperiodic requirement, the most common combination being the need for cold followed by long photoperiods. This leads to flowering in late spring or early summer. There are also cases where vernalization can substitute for, or modify, a photoperiodic response by reducing the critical photoperiod or causing plants to become indifferent to photoperiod (Wilkins, 1984).

Since vernalization requires both carbohydrates, as an energy source, and oxygen, it is unlikely that it will result simply from the absence of an inhibitory reaction at low temperatures. De-vernalization suggests that vernalization probably does not result in the production of a transmissible stimulus, but leads to the development of a localized group of vernalized cells at a certain stage of development. The vernalized condition is a property that is retained in all daughter cells resulting from divisions of vernalized

promeristem cells, unless de-vernalization occurs. New proteins which appeared in winter wheat during a cold treatment resembled those that occurred in a spring cultivar after vernalization (Wilkins, 1984). The use of inhibitors gave further evidence that vernalization is essential for the appearance of the new proteins. Vernalization appears to involve relatively stable changes and once the meristematic tissue has been fully vernalized, the change appears to be transmitted by cell lineage (Wareing & Phillips, 1978). This conclusion may imply that the vernalization stimulus is transmitted through some self replicating cytoplasmic organelle, but it is also possible that certain genes become activated during vernalization and that this change is transmitted to the daughter nuclei during mitosis. (Wareing & Phillips, 1978)

Nakanishi and Fujii developed a biochemical assay system in 1992 for detecting the early stage of flowering. Peroxidase isozymes in shoot apices of induced and non-induced Pharbitis nil plants were analysed using PAGE. They concluded that, as the appearance of cationic isozymes of peroxidase was observed in induced shoot tips cultured in vitro, as well as in intact plants, their peroxidase assay system can be used for the detection of flower-inducing or promoting factors in vitro.

The cold requirement may be either dominant or recessive, which implies positively acting or inhibiting genes. At times the difference is monogenic, and at other times polygenic. As vernalization is gene-determined, it is implied that the mechanism of action starts with the basic material of inheritance, the DNA (Wellensiek, 1965).

Up till now it has generally been accepted that the low temperatures are perceived by the growing tips (Wellensiek, 1965). Not all growing tips perceive the cold, as was described by Chouard (1960) and co-workers, where the age and size of the growing points determined whether they could be vernalized. The first step in an attempt to understand the mechanism of vernalization can thus be defined as: "the influence of low temperature on mitotic cells" (Wellensiek, 1965). Wellensiek here probably meant the differentiation of meristematic cells.

An example of a perennial plant which shows both vernalization and photoperiodic responses, is L. perenne (perennial ryegrass). In this species, flower primordia are initiated in response to winter chilling, but long photoperiods are required for the emergence of the inflorescence. Elongation of the flowering stem does not commence until the photoperiod exceeds 12 hours. The new tillers which emerge during spring and summer are unvernalized and remain vegetative throughout the growing season, until the following winter. Several genes appear to be involved in the inheritance of chilling responses in L. perenne (Wareing & Phillips, 1978).

Although recent developments using SDS-polyacrylamide gel electrophoresis have allowed varieties to be identified by seed using seed protein banding patterns, relative heading date "remains the single most important character for discriminating between ryegrass varieties in field trials" (Halligan et al., 1991). This does not hold true under South African conditions, although it is apparently successful in Europe.

Purvis (1948 and unpublished) carried out some experiments in which the main shoots of vernalized rye plants were removed and the tillers arising thereafter were cut off, as reported by Schwabe (1954). The plants produced tillers which flowered normally, a result which demonstrated that even tillers arising from axillary buds not yet visible at the time of vernalization behaved as if they had been vernalized. Schwabe (1954), states that there is overwhelming evidence that the photoperiodic stimulus is transmissible, but very few experiments have been carried out on the possibility of transport of the vernalization stimulus. Such translocation is the only evidence existing for the material character of the hypothetical flowering and vernalizing substances. This, however, may not always be the case and especially in L. multiflorum under South African conditions. Where some grazing takes place it may be of tremendous importance.

In most species, flower initiation is influenced by a variety of environmental factors. Each is most effectively perceived by particular plant parts which may be either leaves or shoot apices or roots. The various plant parts exchange a number of signals. When the environmental conditions are favourable for flowering, it causes target shoot meristems to change from vegetative to reproductive growth and morphogenesis. In most plants, an

interaction between several factors, most often photoperiod, temperature and irradiance, results in exogenous control of flower initiation. Other factors, such as mineral nutrition, water stress, etc., might also interplay with the above factors, although their roles seem secondary (Bernier et al., 1981 vol.I). Photoperiod and irradiance are mostly perceived by expanded leaves, mineral nutrition and water stress by the roots. It is presumed that all plant parts perceive temperature (Bernier, 1992). Classical evidence, supported by recent results, indicates that low temperatures which promote flowering, are perceived by the shoot apex (Arumuganathan et al., 1991). Different promotive and inhibitory exogenous factors may act at different sites within the plant. When these sites are not in the places where flowers will be formed, there is a requirement for long distance transmission of signals. Long distance signals move essentially in the xylem and phloem sap. Floral signals produced in the leaves, as a result of favourable photoperiods, are transported, with the bulk of the assimilates, in the phloem. Any influence exerted by the roots in the flowering process is presumably transmitted in the xylem sap. Analyses in changes in sap composition at critical times during the floral transition should therefore be undertaken (Bernier et al., 1981 vol. I & II). In the SDP (short day plant) Pharbitis nil, flowering of apices excised from non-induced plants and grown in vitro is promoted by the addition of phloem sap from induced cotyledons to the culture medium (Bernier, 1992).

Meristem tip culture is used as a means of vegetative propagation and also to eradicate viruses from vegetatively propagated species. Although it has been of greatest interest in horticultural crops, the technique could be usefully extended to pasture plants, particularly grasses and clovers. A culture medium, capable of producing plantlets from meristem tips in L. multiflorum, was developed primarily for virus elimination by Dale in 1975. Another interesting application of meristem tip culture would be in studies on the physiology of flowering. "This in vitro approach to the study of plant development is being used in a number of species and could be usefully extended to grasses" (Dale, 1975).

Generally, roots do not seem to play a role in photoinduction, since the LDP (long day plant) L. multiflorum can be photoinduced in the absence of any visible root or root primordium (Evans, 1969, sited in Bernier et al., 1981 vol. II). The presence of the epicotyl in the absence of roots leads to early flowering and the presence of roots in the

absence of the epicotyl leads to vegetative growth (Miginiac & Lacombe, 1973, sited in Bernier et al., 1981 vol. II). In several species, terminal inflorescences are apparently never produced even with strong floral induction. This is very important in agricultural crops, since plant stature and flower position is strongly correlated (Bernier et al., 1981 vol. II). Axillary buds located in the apical part of the stem, flower rapidly, while those located in the basal part of the stem do so far more slowly, only after a much greater number of leaves are produced (Bernier et al., 1981 vol. II). Apical and basal axillary buds flower at approximately the same time and after having produced the same number of leaves when isolated from the mother plant (McDaniel, 1976, sited in Bernier et al., 1981 vol. II).

Meristems are not merely passive receptors for the floral stimuli. Young leaves or buds might be required in addition to mature leaves for photoinduction to proceed. Correlative influences in the control of initiation of flowering may exist, because terminal meristems of shoot and other axillary meristems may remain vegetative (Bernier et al., 1981 vol. II).

In several cold requiring plants, low temperatures affect bolting and flower initiation (Bernier et al., 1981 vol. II). An early and general feature of the onset of reproductive growth is the early formation of axillary buds (Bernier et al., 1981 vol. II). One of the earliest signs of reproductive transition is an increased growth of young internodes. This "bolting" (rapid stem elongation) is most obvious in plants with a rosette growth habit at the vegetative stage. Stem elongation appears as a rather general effect of induction, but it would be incorrect to conclude from this that flowering plants are taller than vegetative ones. Sometimes the initial growth promotion is followed by a very strong inhibition. Although tiller development and flower initiation are usually associated processes, they can be separated in many plants (Bernier et al., 1981 vol. II).

In grasses, such as Lolium, the shoot apex develops an elongated cone during vegetative growth with two ranks of alternating unexpanded leaf primordia, which appear as a single ridge (Evans, 1969, sited in Bernier et al., 1981 vol. II). When these plants enter the reproductive stage, small bulges appear in the axils of all leaf primordia present at the time, resulting in a "double-ridge" appearance. These "bulges" are usually first formed in

the middle of the elongated apex and proceed acro- and basipetally. The axillary primordia have a far greater growth rate than the subtending leaf primordia and develop into spikelets. Most of the changes noted in Lolium occurred as early in the leaf primordia as they did in the meristem itself. The changes in leaf primordia were relatively smaller than those observed in the meristem, although still highly significant (Bernier et al., 1981 vol. II). An increased rate of leaf initiation, preliminary to floral initiation, is widespread and was also observed in the LDP Lolium (Evans, 1960, sited in Bernier et al., 1981 vol. II). A close relationship apparently exists between the transition to flowering and the promotion of leaf production, although the latter process does not cause flower formation. Subminimal induction in young Lolium plants also increases the rate of leaf inception without any sign of spikelet production (Evans, 1960, sited in Bernier et al., 1981 vol. II).

Elongated vegetative meristems are mostly found in grasses and aquatic plants. There is a marked "doming" of the meristem at the initiation of the first flower primordia or shortly preceding it (Bernier et al., 1981 vol. II). This increase in height relative to width occurs in most plants, even in the grasses which already possess an elongated vegetative meristem (Pedurand, 1969, sited in Bernier et al., 1981 vol. II). Correlations between meristem size and inflorescence development are reported for Lolium, and it has been suggested that flower initiation is an inevitable consequence of exceeding a critical size of the apical meristem dome (Evans, 1960, sited in Bernier et al., 1981 vol. II). Meristem enlargement may also occur without flower initiation, but its size in relation to that of the primordia at initiation could well be of decisive importance (Bernier et. al., 1981 vol. II).

Flower formation can be preceded, and is usually accompanied, by several macroscopic changes:

- increased elongation of young internodes
- precocious initiation of axillary buds
- increased growth rate of leaves
- change in the shape of leaves
- increased rate of initiation of leaves and primordia
- doming and enlargement of the meristem
- increased phyllotaxis

It can be concluded that the techniques of cell and molecular biology can play an important role in the study of the physiology of flowering.

1.3 Morphological study

The morphology of the induction of flowering should be based on a knowledge of the effect of environmental conditions on the rate of growth and the pattern of morphological development.

Mitchell, (1953, vol. I) stated that variations in light and temperature resulted in large differences in the pattern of growth and the quantity of tissue produced by the plants. In his study, Mitchell used the appearance of leaves on the main stem as a physiological time scale for measuring the vegetative age of plants and found that until a leaf on any stem had developed, its axillary bud could not grow out to a tiller.

From a comparison of tables 1 and 2 it can be seen that, in Mitchell's (1953) experiment, optimum growth conditions might have resulted in a higher rate of tillering than found in this study (table 2). There is however a large amount of variation from plant to plant.

Table 1

Daily increase in the number of tillers per plant in Lolium spp.(Mitchell, 1953 vol. I)

Age of plants in days	Number of tillers formed
8	3
16	5
24	7
32	12
40	19

Raising the light intensity, or lowering the temperature, or both, results in an increased rate of tillering (Mitchell, 1953 vol.I), while partial defoliation can inhibit the formation of axillary buds (Mitchell, 1953 vol.II).

In the present study, photographs were taken of L. multiflorum plants to show the base of the tillers and the root formation of plants at two, four, seven and twelve months after

germination (figure 1). There was not a marked difference between the development of Italian and Westerwolds type plants kept under different conditions, ie. long days and temperatures ranging from a minimum of 15°C to a maximum of 30°C. The photographs taken are from Italian type plants, cv. Tetrone. Variation between plants within a cultivar was also greater than expected. Photographs were also taken, under the stereo microscope, of young tillers, to show their development, as well as their orientation to the main tiller (figure 2). The number of tillers that have developed on each plant in figure 1, can be seen in table 2 against the ages of the plants. As Mitchell found in 1953, it was also found in this study that a new tiller developed from an axillary bud only after at least one mature leaf was present on an existing tiller.

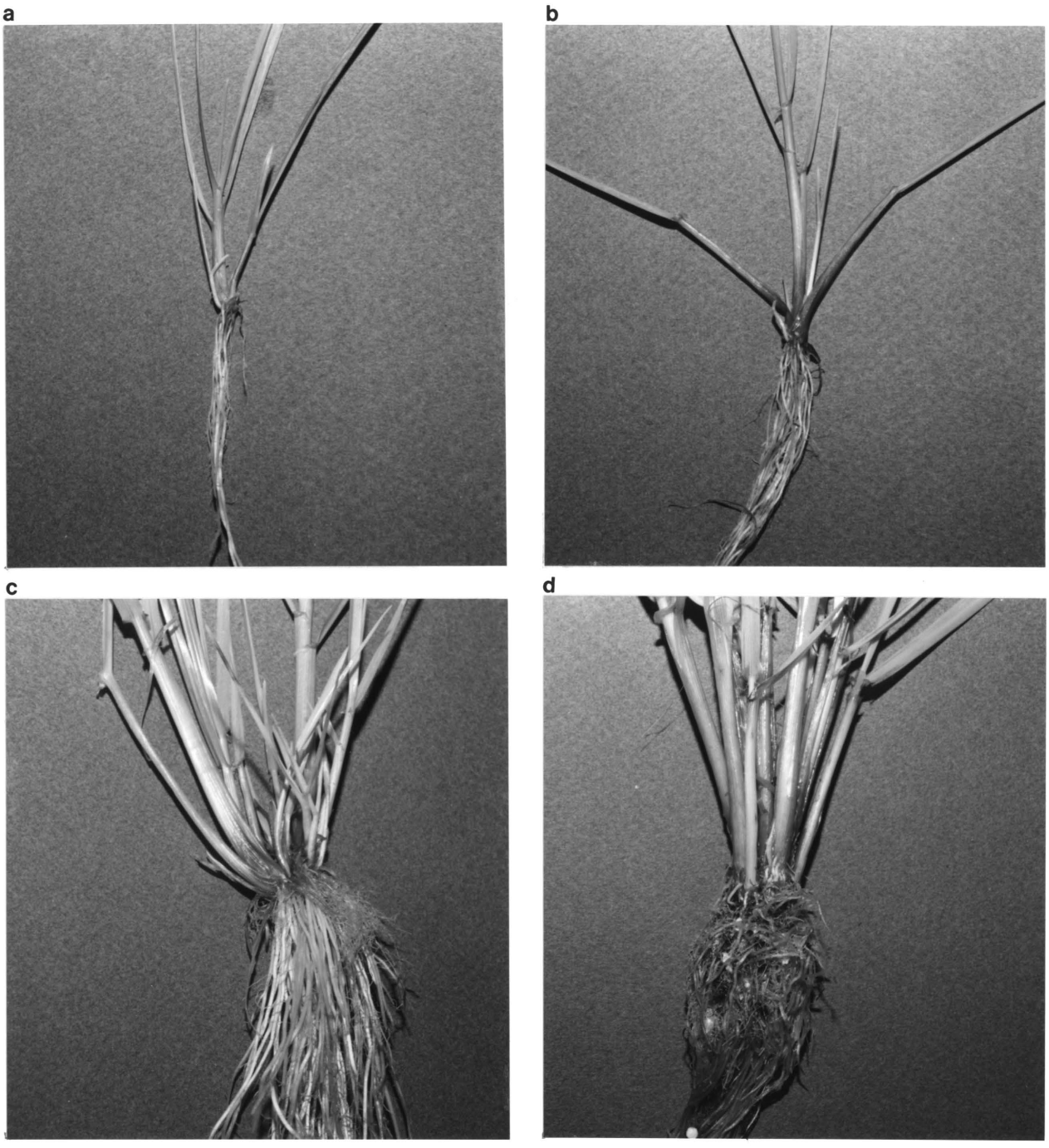

Figure 1 Tillering and root formation shown for L. multiflorum plants 2 months (a), 4 months (b), 7 months (c), and 12 months (d) after germination

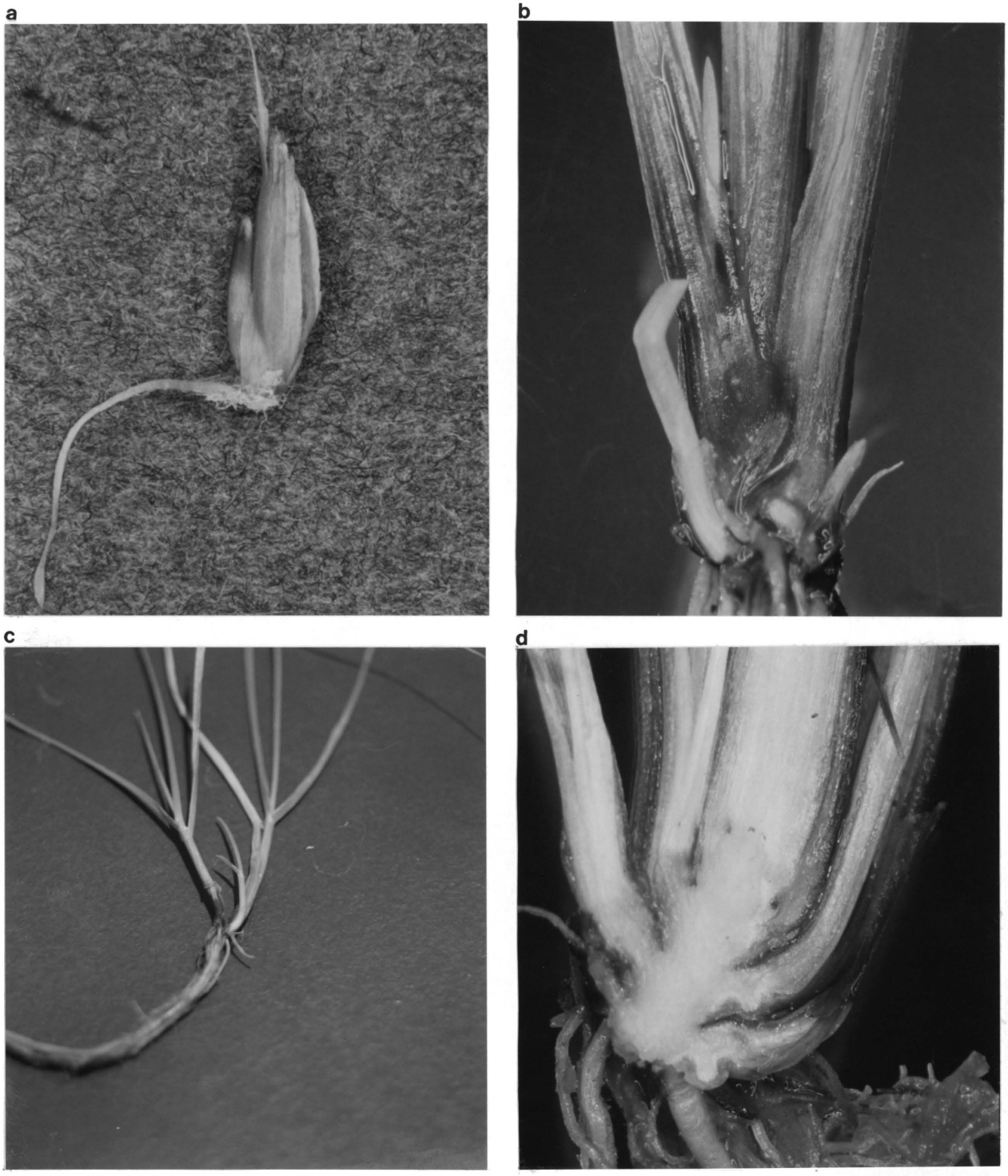

Figure 2 The development (a and b) and orientation (c and d) of tillers of L. multiflorum, as seen under the stereo microscope

Table 2

The rate of tillering in L. multiflorum during vegetative development

Age of plants in months	Number of tillers
2	3
4	5
7	9
12	14

From figure 2 it is evident that the apical meristem in L. multiflorum is situated just above the roots, in the base of the tiller, which is in the upper layer of the soil. For this study, this is a very important fact, as grazing should therefore not remove the apical meristem. Temperature of vernalization might also imply the temperature of the upper level of the soil. The fact that Mitchell (1953) also stated that partial defoliation can inhibit the formation of axillary buds and therefore tillering, can also have implications for the grazing management of this pasture grass.

CHAPTER 2

VERNALIZATION OF SEED, GENETS AND RAMETS

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2.1 Introduction

Some of the L. multiflorum cultivars commercially available in South Africa, consist of mixtures of Italian and Westerwolds types. These two types, however, seem to have different requirements for the induction of flowering and thus seed production. Italian type plants supposedly require a period of winter cold (vernalization), followed by an increase in daylength (photoperiod), whereas Westerwolds type plants seem to require only an increase in daylength.

The local cultivar Midmar, which contains 20% Italian and 80% Westerwolds type plants, has become very popular due to its wide adaptability in areas with different climatic conditions to the area where it was developed, ie. the Natal Midlands. Although the legislated close-down date for seed production units of both Italian and Westerwolds cultivars is 15 October, in many seed producing areas closing down at such a late date may result in the removal of reproductive tillers. It is possible that Italian type plants will form new tillers which will remain vegetative in the absence of cold and may result in a reduced seed yield. Westerwolds type plants will produce new tillers which will go into the reproductive phase. Consequently cultivars that are mixtures of Italian and Westerwolds types, ie. Midmar, are in danger of undergoing genetic shift towards the Westerwolds type if closed down too late. Thus the different requirements of Italian and Westerwolds plants for the induction of flowering have important implications with respect to seed yields, the maintenance of genetic stability and composition. The OECD and the South African Seed Certification Scheme puts great emphasis on the maintenance of genetic stability and composition of cultivars. It is, therefore, important that the composition for which a cultivar was bred, is maintained in order to maintain its dry matter production characteristics.

Because of the various climatic regions in South Africa, this "problem" with respect to these "mixed" cultivars is unique to South Africa. Specific "problem areas" for seed production will probably include the temperate coastal regions, where the plants might not get a vernalization period that is cold or long enough. These areas should then preferably be avoided for seed production of cultivars containing Italian type plants. Correct management of seed production units is therefore of vital importance if genetic shift and reduced seed yields are to be avoided.

To examine the possibility of genetic shift, it is important to know if the vernalization stimulus is translocated through the plant and/or retained in the vernalized tissue. If translocation does occur, new tillers arising after the removal of vernalized tillers should still be induced for flowering. This, however, may not always be the case. Where some grazing takes place it may be of tremendous importance.

No references could be found on the translocation of the vernalization stimulus in L. multiflorum, but Bernier (1992) reported an experiment carried out by Purvis on rye plants. In this experiment the main shoots were removed after vernalization and tillers arising after that were cut off. New tillers were produced which flowered as if they had been vernalized. These new tillers arose from axillary buds not yet visible at the time of vernalization. Schwabe stated in 1954 that very few experiments had been carried out on the possibility of transport of the vernalization stimulus in different species.

The aim of this study is to determine the vernalization requirements of L. multiflorum cultivars used in the R.S.A., in an attempt to predict suitable areas for seed production of cultivars containing Italian type plants in such a way as to minimize the possibility of genetic shift. The induction of flowering of vernalized tillers, as well as that of tillers arising after vernalization, will also be examined to establish whether grazing of Italian type plants in seed production units can be recommended after vernalization occurred. The shoot apices of some of these tillers will also be examined to see if the reproductive phase can be recognised at a very early stage.

2.2 Materials and methods

2.2.1 **Vernalization of seed**

Italian ryegrass, here represented by the tetraploid cultivar Tetrone, needs vernalization, followed by a long photoperiod, for the initiation of flowering as well as for flowering itself.

Imbibed seed of Tetrone (seed lot #LM 61 J) was submitted to four temperature treatments for three weeks in the dark in growth chambers. These four temperature treatments were -1°C, 4°C, 8°C and 11°C, the latter being the control. Seed of a tetraploid Westerwolds cultivar, Billion (seed lot #LM 51 I), was also included in the control. The four treatments were replicated four times in a randomized block design. Twenty seeds were put on filter paper in each petri dish and 3 ml of distilled water added. These were then closed to prevent loss of moisture and put in growth chambers at the temperatures mentioned above.

After three weeks, the seed was germinated on a Jacobsen water table. After germination (5-7 days later), the seedlings were planted in Hygromix medium in seedling trays. These were then put in the glasshouse at long photoperiods (12+ hours) and temperatures between 20°C and 30°C, so that vernalization was unlikely to occur and the photoperiods were longer than 12 hours to promote flowering. As soon as the seedlings were established, after two weeks, they were transplanted into 40 pots, with three plants per pot, and kept under the same conditions as before. An experimental unit was one pot, consisting of three plants. Because of variation in the environment in the glasshouse as a result of air movement, irrigation, etc., the pots were rotated within replications on a weekly basis.

It is postulated that grazing of seed production units after vernalization can result in a change in the composition of the Italian and Westerwolds types of a "mixed" cultivar. To examine this possibility, half of the number of plants in each treatment above was cut to five centimetres as they were transplanted into the pots. The other half of the plants served as the control.

Inflorescences were counted weekly, starting when the first inflorescence appeared.

2.2.2 **Vernalization of genets (plants, zygotic origin)**

Seed of two tetraploid cultivars, Tetrone (Italian) and Billion (Westerwolds) was germinated on a Jacobsen water table. After six days the seedlings were planted in Hygromix medium in seedling trays and kept in the glasshouse at long photoperiods (12+ hours) and temperatures of 20°C to 30°C. When the seedlings were three weeks old, they were taken from the glasshouse and treated in growth chambers for three weeks. Four different temperature regimes were used (table 3) and they were each divided into short photoperiods (9 hours of light, 15 hours of darkness) and long photoperiods (14 hours of light, 10 hours of darkness). Each of the treatments was replicated three times in a randomized block design.

Table 3 The four temperature regimes used in the treatment of L. multiflorum plants with both cultivars (Tetrone and Billion) included in the control

NIGHT TEMPERATURES (°C)	**DAY TEMPERATURES (14 HOURS) (°C)**	**DAY TEMPERATURES (9 HOURS) (°C)**
4	13	13
4	23	23
10	13	13
10	23	23 (control, Tetrone)
10	23	23 (control, Billion)

Only Tetrone was treated at all temperature regimes, except for the control, where the Westerwolds cultivar, Billion, was also included.

After treatment, the plants were transplanted into 60 pots, three plants per pot, each pot being an experimental unit. These were then returned to the glasshouse at warmer temperatures (20°C - 30°C) and long photoperiods (12+ hours). Because of variation in the growth chambers due to air movement and the positioning of the lights, the seedling trays were rotated daily. In the glasshouse, too, pots were rotated weekly within replications.

There is a possibility of a change in the composition of a "mixed" cultivar in terms of the relative proportions of Italian and Westerwolds types due to the grazing of production units, intended for seed production later on, after vernalization. Therefore half of the number of plants in each treatment above was cut to five centimetres after treatment. The other half of the plants served as a control.

Inflorescences were counted weekly, starting when the first inflorescence appeared.

2.2.3 **Vernalization of ramets (clonal plants, vegetative origin)**

The tetraploid Italian cultivar, Tetrone, was again used in this experiment. Different vernalization conditions were examined, as it was suspected that the duration of the vernalization period should be longer than the period used previously. Because of the possibility of seed contamination and also variation within the cultivar, ramets of each plant were subjected to different periods of vernalization.

Seedlings of the cultivar Tetrone were grown for two months under non-inductive conditions in the glasshouse. Forty plants were then cloned into three plantlets each, consisting of one to three tillers. Each one of these ramets was planted in a 10 cm pot in Hygromix medium and kept for one week in the glasshouse, at non-inductive conditions, to establish. The ramets of the 40 original plants were then put into four growth chambers. The 30 ramets of plants 1-10 were put into chamber A1 and the 30 ramets of plants 11-20 into chamber A2. Growth chambers A1 and A2 were both set for a day temperature of 13°C and a night temperature of 4°C. The photoperiod (daylength) resembled that of mid-winter, ie. nine hours. The ramets of the other 20 plants were put into chambers B1 and B2. 30 Ramets of plants 21-30 went into chamber B1 and the 30 ramets of plants 31-40 went into chamber B2. The temperature in growth chambers B1 and B2 was 23°C during

the day and 4°C at night. The photoperiod was again nine hours.

One of the three ramets of each of the 40 plants was taken out of the growth chambers after three, five and 12 weeks. Thus, the three ramets of each of the 40 original plants were subjected to the same temperature regimes, but different periods of vernalization, so that comparisons could be made, within temperature regimes, between the vernalization periods of three, five and 12 weeks. Thus, with genetically identical material subjected to the same temperature regimes, it should be possible to establish the most suitable period of cold for vernalization.

After the ramets were taken from the growth chambers, they were kept in the glasshouse at temperatures ranging from a minimum night temperature of 16°C to a maximum day temperature of 35°C. Because of irrigation and air movement in the glasshouse, and air movement and the positioning of the lights in the growth chambers, pots were rotated on a regular basis. As control for the experiment 20 Tetrone plants were kept in the glasshouse at non-inductive conditions for the duration of the experiment.

Inflorescences were counted every week, starting when the first inflorescence appeared.

2.2.4 **Examination of shoot apices**

A test sample was taken from the experiment described above in 2.2.3. Twenty four plants were tested, four plants from each treatment. Of each plant, an "old" and a "young" tiller were examined. An old tiller being one of the tillers present at the start of vernalization and a young tiller being any tiller which appeared during or after vernalization.

The shoot apex of a young and an old tiller of each plant included in the test sample was excised and examined under the microscope for the "double-ridge" stage, which indicates that the apex has entered the reproductive phase. Small "bulges" are formed in the axils of all leaf primordia present at the time. The tillers were marked as "reproductive" and "vegetative", according to the phase observed in the shoot apex.

To establish whether translocation of the vernalization stimulus took place in this case, old and young tillers, as defined in 2.2.4, were compared, not only by looking at their shoot apices, but also by monitoring if any young tillers were forming inflorescences.

2.3 Results

2.3.1 **Vernalization of seed**

Inflorescences first appeared sixteen weeks after the seed was treated and were counted weekly until twenty seven weeks after treatment. The results are presented as follows:

Table 4 The cumulative number of inflorescences formed twenty one weeks after treatment of seed of L. multiflorum

TREATMENT	*REP. 1	REP. 2	REP. 3	REP. 4
ITALIAN -1°C (TETRONE)				
CUT	0	0	0	0
INTACT	0	0	0	0
ITALIAN 4°C (TETRONE)				
CUT	0	0	0	0
INTACT	0	1	0	0
ITALIAN 8°C (TETRONE)				
CUT	0	0	0	0
INTACT	0	0	0	0
ITALIAN 11°C (TETRONE)				
CUT	0	0	0	0
INTACT	0	0	0	0
WESTERWOLDS 11°C (BILLION)				
CUT	3	0	2	0
INTACT	0	8	0	0

*Replication

Table 5 The cumulative number of inflorescences formed twenty seven weeks after treatment of seed of L. multiflorum

TREATMENT	*REP. 1	REP. 2	REP. 3	REP. 4
ITALIAN -1°C (TETRONE)				
CUT	0	0	0	0
INTACT	0	0	0	0
ITALIAN 4°C (TETRONE)				
CUT	0	0	0	0
INTACT	0	**1**	0	0
ITALIAN 8°C (TETRONE)				
CUT	0	0	0	0
INTACT	0	0	0	0
ITALIAN 11°C (TETRONE)				
CUT	0	0	0	0
INTACT	0	0	0	0
WESTERWOLDS 11°C (BILLION)				
CUT	**9**	0	**10**	0
INTACT	0	**15**	0	**4**

*Replication

As can be seen in the tables above, only one inflorescence was formed in the Italian cultivar, Tetrone, in one replication only, while the Westerwolds cultivar Billion, as control, flowered in each replication.

Tetrone seed treated for three weeks at 4°C and left intact produced only one inflorescence, and only in one replication. This indicated possible contamination of seed, rather than successful treatment.

Billion, included as a control at 11°C, formed inflorescences in cut plants as well as plants that were left intact. In all the replications some flowering occurred. This indicated that the photoperiod was sufficient for the formation of inflorescences.

The reasons for the unsuccessful treatment of Tetrone can be the result of three possibilities. Firstly, the temperatures used might not have been cold enough for vernalization to occur. This is doubted, though, as Wareing and Phillips (1978) stated that, for the majority of species, the most effective temperatures for vernalization are just above freezing, but temperatures from -1°C to 9°C are equally effective. Secondly, the photoperiod could have been insufficient (too short) for the formation of inflorescences. This possibility can be ruled out, however, as the Westerwolds cultivar, Billion, which only needs a certain photoperiod for the induction of flowering, did form inflorescences, indicating that the photoperiod was long enough. Lastly, it can be concluded that Tetrone, and all Italian type plants, can probably be vernalized only as plants and not as imbibed seeds. Wareing and Phillips (1978) reported that, generally, species that can be vernalized at the seed stage do not require vernalization again at the plant stage, whereas those which can only be vernalized at the plant stage have an obligate cold requirement.

2.3.2 **Vernalization of genets**

The first inflorescences, those of the control (Billion), appeared nine weeks after treatment. Inflorescences were counted weekly, until twenty weeks after treatment.

Table 6 The cumulative number of inflorescences of L. multiflorum formed ten weeks after treatment of genets

NIGHT/DAY TEMPERATURE (°C)	[1]PHOTO= PERIOD	INTACT/ CUT	[2]REP. 1	REP. 2	REP. 3
4/13	LONG	INTACT	0	0	0
		CUT	1	0	0
	SHORT	INTACT	0	0	0
		CUT	0	0	0
4/23	LONG	INTACT	0	0	0
		CUT	0	1	0
	SHORT	INTACT	0	0	0
		CUT	0	0	0
10/13	LONG	INTACT	0	0	0
		CUT	0	0	0
	SHORT	INTACT	0	0	0
		CUT	0	0	0
10/23 (ITALIAN)	LONG	INTACT	0	0	0
		CUT	0	0	0
	SHORT	INTACT	0	0	0
		CUT	0	0	0
10/23 (WESTERW.)	LONG	INTACT	3	0	0
		CUT	0	0	0
	SHORT	INTACT	0	0	0
		CUT	5	1	1

[1]Long photoperiod=14h, short photoperiod=9h [2]Replication

Table 7 The cumulative number of inflorescences of L. multiflorum formed fifteen weeks after treatment of genets

NIGHT/DAY TEMPERATURE (°C)	[1]PHOTO= PERIOD	INTACT/ CUT	[2]REP. 1	REP. 2	REP. 3
4/13	LONG	INTACT	0	0	0
		CUT	7	0	0
	SHORT	INTACT	0	0	0
		CUT	0	0	0
4/23	LONG	INTACT	0	0	0
		CUT	0	33	0
	SHORT	INTACT	0	0	0
		CUT	0	0	0
10/13	LONG	INTACT	0	0	0
		CUT	0	0	0
	SHORT	INTACT	0	0	0
		CUT	0	0	0
10/23 (ITALIAN)	LONG	INTACT	0	0	0
		CUT	2	0	0
	SHORT	INTACT	0	0	0
		CUT	0	0	0
10/23 (WESTERW.)	LONG	INTACT	18	6	0
		CUT	0	0	0
	SHORT	INTACT	0	0	0
		CUT	9	1	6

[1]Long photoperiod=14h, short photoperiod=9h [2]Replication

Table 8 The cumulative number of inflorescences of L. multiflorum formed twenty weeks after treatment of genets

NIGHT/DAY TEMPERATURE (°C)	[1]PHOTO= PERIOD	INTACT/ CUT	[2]REP. 1	REP. 2	REP. 3
4/13	LONG	INTACT	0	0	0
		CUT	26	0	0
	SHORT	INTACT	0	0	0
		CUT	0	0	0
4/23	LONG	INTACT	0	0	0
		CUT	0	63	0
	SHORT	INTACT	0	0	0
		CUT	0	0	0
10/13	LONG	INTACT	0	0	0
		CUT	0	0	0
	SHORT	INTACT	0	0	0
		CUT	0	0	0
10/23 (ITALIAN)	LONG	INTACT	0	0	0
		CUT	2	0	0
	SHORT	INTACT	0	0	0
		CUT	0	0	0
10/23 (WESTERW.)	LONG	INTACT	43	12	0
		CUT	1	0	0
	SHORT	INTACT	0	0	0
		CUT	16	1	8

[1]Long photoperiod=14h, short photoperiod=9h [2]Replication

Only three Italian type plants flowered, two of which abundantly and in only one of the replications, resulting in the formation of numerous inflorescences. At close examination of the morphology of these plants, it was evident that they were possibly tetraploid Westerwolds type plants, possibly the result of seed contamination and, therefore, it did not appear as a positive response to the treatments. While examining these plants, it was noted that the remaining Italian type plants in the experiment (141) differed slightly in their morphology, which could mean that genetic variation within this cultivar, Tetrone, was greater than expected when it was included in the experiment.

The fact that the Westerwolds type plants, included as a control, flowered, indicated that the photoperiod was long enough for the formation of inflorescences. Plants flowered in all three replications.

It can also be noted in the tables above that inflorescences occurred mainly on plants that were cut after treatment. Contrary to the statement of Mitchell (1953) (see page 17) it is believed that, as the shoot meristem, where vernalization is perceived, was left intact, increased flowering might also be due to increased tillering, caused by cutting. Plants were cut in order to find out whether the vernalization stimulus is translocated through the plant or not. This proved unsuccessful, as the shoot meristems were still in the basal part of the tiller and were left intact.

One of the reasons for the unsuccessful vernalization treatments could be the fact that the temperature regimes used were still not cold enough, though it was expected that a day/night temperature of 4/13°C should have been cold enough for vernalization to occur.

The long or short photoperiods during the treatments did not seem to have any significant effect, since only Westerwolds type plants flowered, both in long and short photoperiods.

2.3.3 **Vernalization of ramets**

Inflorescences formed by the treated ramets were counted weekly for a total of twelve weeks. The raw data is presented in tables 9-11.

Table 9 The cumulative number of inflorescences formed by L. multiflorum (Tetrone) ramets after three weeks of treatment

TEMPERATURE (NIGHT/ DAY)	PLANT NUMBER	INFLORESCENCES FORMED	TEMPERATURE (NIGHT/ DAY)	PLANT NUMBER	INFLORESCENCES FORMED
4/13°C	1/1	0	**4/23°C**	21/2	0
	2/1	0		22/3	0
	3/2	0		23/1	0
	4/2	0		24/3	0
	5/2	0		25/1	0
	6/3	0		26/3	0
	7/1	0		27/3	0
	8/3	0		28/1	0
	9/2	0		29/2	0
	10/2	0		30/2	0
	11/2	0		31/2	0
	12/1	0		32/1	0
	13/3	0		33/2	0
	14/1	0		34/2	0
	15/1	0		35/2	0
	16/2	0		36/1	0
	17/1	0		37/1	0
	18/3	0		38/1	0
	19/1	0		39/3	0
	20/2	0		40/2	0

Table 10 The cumulative number of inflorescences formed by L. multiflorum (Tetrone) ramets after five weeks of treatment

TEMPERATURE (NIGHT/ DAY)	PLANT NUMBER	INFLORESCENCES FORMED	TEMPERATURE (NIGHT/ DAY)	PLANT NUMBER	INFLORESCENCES FORMED
4/13°C	1/2	0	**4/23°C**	21/3	0
	2/3	0		22/1	0
	3/1	0		23/3	0
	4/1	0		24/2	0
	5/3	0		25/2	0
	6/1	0		26/2	0
	7/3	0		27/2	0
	8/1	0		28/2	0
	9/1	0		29/1	0
	10/1	0		30/3	0
	11/1	**1**		31/1	0
	12/2	**1**		32/2	0
	13/2	**1**		33/3	0
	14/2	0		34/3	0
	15/3	0		35/1	0
	16/1	**1**		36/2	0
	17/2	0		37/3	0
	18/2	0		38/2	0
	19/3	0		39/1	0
	20/1	**1**		40/1	0

Table 11 The cumulative number of inflorescences formed by L. multiflorum (Tetrone) ramets after 12 weeks of treatment

TEMPERATURE (NIGHT/ DAY)	PLANT NUMBER	INFLORESCENCES FORMED	TEMPERATURE (NIGHT/ DAY)	PLANT NUMBER	INFLORESCENCES FORMED
4/13°C	1/3	0	**4/23°C**	21/1	0
	2/2	0		22/2	0
	3/3	0		23/2	0
	4/3	0		24/1	0
	5/1	0		25/3	0
	6/2	0		26/1	0
	7/2	0		27/1	0
	8/2	0		28/3	0
	9/3	0		29/3	0
	10/3	0		30/1	0
	11/3	**1**		31/3	0
	12/3	**3**		32/3	0
	13/1	**3**		33/1	0
	14/3	**1**		34/1	0
	15/2	**2**		35/3	0
	16/3	**3**		36/3	0
	17/3	**3**		37/2	0
	18/1	**1**		38/3	0
	19/2	**2**		39/2	0
	20/3	**3**		40/3	0

The ramets that were treated for three weeks did not form any inflorescences. Ramets treated for five weeks, however, formed the first inflorescences four weeks after the treatment, while ramets treated for twelve weeks formed their first inflorescences during the twelfth week of the treatment. It therefore seems that cold treatment combined with another stimulus could induce flowering even without the occurrence of high temperatures.

No inflorescences were formed by the ramets treated at a temperature regime of 4°C at night and 23°C during the day. Due to a faulty time switch on the lights of one of the growth chambers, that particular growth chamber had continuous lighting for an unknown period of time. As can be seen in table 10, plant numbers 11-13, 16 and 20 formed inflorescences after five weeks of treatment, while plant numbers 11-20, which were also treated at the same temperature, but for twelve weeks, all formed inflorescences. All these plants that did form inflorescences, were treated in the growth chamber with the continuous lighting.

A simple statistical analysis was carried out on the data in order to establish whether the results were significant. As only one of the two temperature treatments was successful, only that particular treatment was included in the analysis. This temperature treatment was split, because of the continuous lighting in one of the growth chambers of this treatment. Consequently only one temperature treatment was tested against the three different periods of treatment in a chi-squared test, which are presented in table 12.

Table 12 The chi-squared test carried out on some of the raw data of the treatment of L. multiflorum (Tetrone) ramets in tables 9-11.

	OBSERVED FREQUENCIES	OBSERVED FREQUENCIES	OBSERVED FREQUENCIES
FLOWERING:	**YES**	**NO**	**TOTAL**
T_1A_2	0	10	10
T_2A_2	5	5	10
T_3A_2	10	0	10
TOTAL	15	15	30

CHI-SQUARED VALUE = 20

TABLED CHI-SQUARED VALUE = 5.991

D.F. = 2

TEST LEVEL = 0.05

APPROXIMATE PROBABILITY = 0

THE FREQUENCIES ARE SIGNIFICANTLY DIFFERENT.

A_2 = night/day temperature of 4/13°C

T_1 = treatment period of three weeks

T_2 = treatment period of five weeks

T_3 = treatment period of twelve weeks

Thus there is a significant dependency between the duration of the treatments and the formation of inflorescences. This indicates a trend for forming more inflorescences over a period of time of cold treatment (vernalization). An example is plant number 20, of which ramets can be seen in figure 3. Number 20/3 produced two more inflorescences after the photographs were taken, as the photographs were taken during the monitoring period. The control, which received no cold treatment, did not form any inflorescences.

To establish whether translocation of the vernalization stimulus takes place, the age of the flowering tillers was also noted during the experiment. Ramets consisted of one to three tillers each. During treatment, particularly the colder temperature regime, a number of new

tillers were formed. By the time the experiment was finished, however, only tillers that were present when treatment started had formed inflorescences, while tillers formed during or after treatment were still in the vegetative phase.

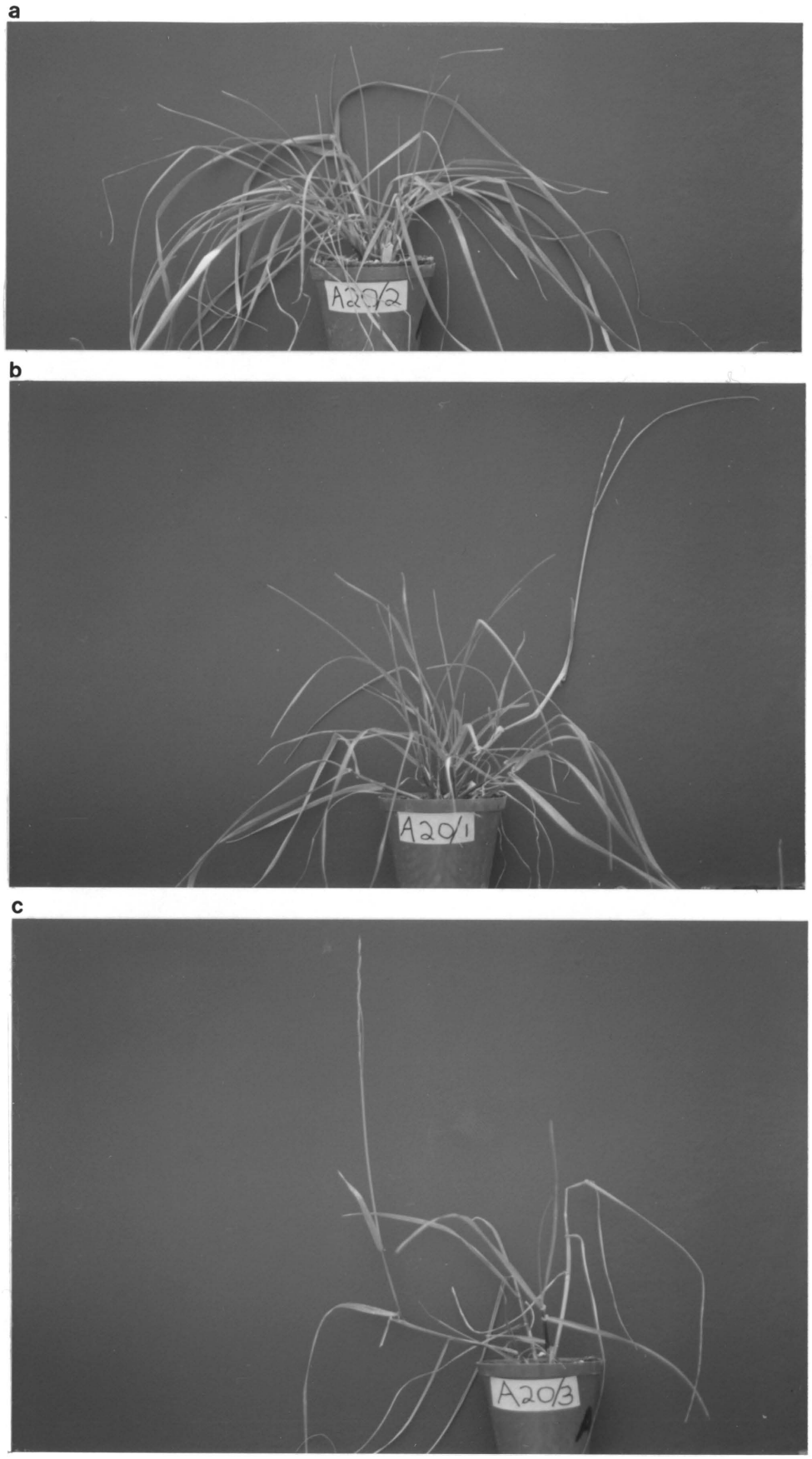

Figure 3 Ramets of plant #20 of L. multiflorum (Tetrone) after (a) 3 weeks, (b) 5 weeks and (c) 12 weeks of treatment

2.3.4 **Examination of shoot apices**

Tillers of the twenty four plants in the test sample were examined under the microscope and photographs were taken of some of the "reproductive" and "vegetative" shoot apices. The results are presented in table 13.

Table 13 Results of the examination of a test sample from ramets of L. multiflorum cv. Tetrone treated in 2.2.3

PERIOD OF TREATMENT	TEMPERATURE (NIGHT/DAY)	PLANT NUMBER	OLD TILLER (*R/V)	YOUNG TILLER (*R/V)
3 WEEKS	4/13°C	6/3	V	V
		11/2	-	V
		12/1	V	V
		20/2	V	V
	4/23°C	21/2	V	V
		25/1	V	V
		39/3	V	V
		40/2	V	V
5 WEEKS	4/13°C	6/1	V	V
		11/1	R	V
		12/2	R	V
		20/1	R	V
	4/23°C	21/3	V	V
		25/2	V	V
		39/1	V	V
		40/1	V	V

*Reproductive/Vegetative

Table 13 (continued)

PERIOD OF TREATMENT	TEMPERATURE (NIGHT/DAY)	PLANT NUMBER	OLD TILLER (*R/V)	YOUNG TILLER (*R/V)
12 WEEKS	4/13°C	6/2	V	V
		11/3	R	V
		12/3	R	V
		20/2	R	V
	4/23°C	21/1	V	V
		25/3	V	V
		39/2	V	V
		40/3	V	V

*Reproductive/Vegetative

From table 13 it can be seen that the "young" tillers examined were all in the vegetative stage. The "old" tillers that were reproductive, were all treated at the same temperature regime of a night/day temperature of 4/13°C, for a period of five and twelve weeks. These results are merely observations and the data were not statistically analysed. The plants represented in this test sample that were in the reproductive phase, later formed inflorescences in 2.3.3. Plants were examined four to six weeks after treatment. Figure 4 shows examples of "reproductive" (a and b) and "vegetative" (c) shoot apices. The "bulges" in the axils of the leaf primordia, which are the spikelet primordia, can be seen in (a) and (b), while only leaf primordia are present in (c).

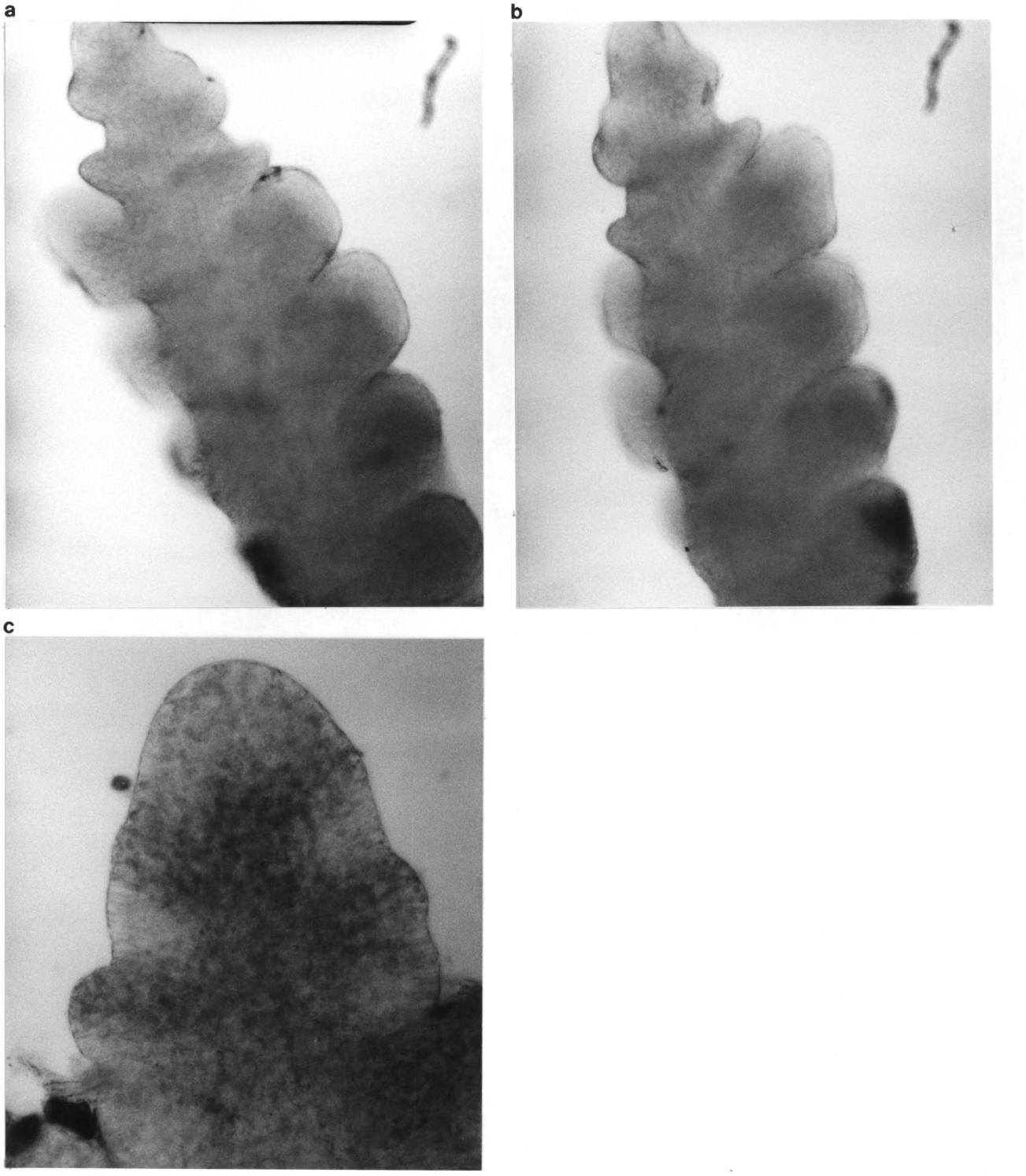

Figure 4 Reproductive (a and b) and vegetative (c) shoot apices of Tetrone ryegrass after four to six weeks of vernalization

The comparison of old and young tillers confirmed the fact that, in this experiment, translocation of the vernalization stimulus did not seem to take place.

2.4 Discussion

2.4.1 **Vernalization of seed**

The implication of these results is that Italian type plants of L. multiflorum can be vernalized only at plant stage and should, therefore, be planted before the winter, in autumn, if a seed crop is required. Planting Italian cultivars in spring results in pastures lasting about eighteen months and flowering during the second summer. Thus the pure Italian type cultivars, like Tetrone, and "mixed" cultivars, like Midmar, should be established in autumn in order to be vernalized as plants during the following winter. Seed would be formed and genetic shift, as well as reduced seed yields, would be avoided, resulting in the maintenance of genetic stability.

2.4.2 **Vernalization of genets**

As no positive results could be obtained from this experiment, it was decided to repeat it, with certain amendments. It was concluded that areas that needed to be tested again, were not so much the temperature regimes, which were suspected to be adequate for vernalization, but the period of time for which the plants were subjected to treatment, ie. dosage of the cold treatment. As there was marked genetic variation within Tetrone, it was decided to subject ramets (clones) to different lengths of treatment in a further experiment.

2.4.3 **Vernalization of ramets**

As discussed in 2.4.2, it is now probable that a temperature regime of 4°C at night and 13°C during the day is adequate for the induction of flowering in L. multiflorum cv. Tetrone. This corresponds with the statement of Wareing and Phillips (1978) that temperatures ranging from -1°C to 9°C are effective for the vernalization of the majority of species. The duration of the cold period should be at least five weeks, according to data from this thesis. There is also a correlation, or rather a dependency, between the duration of the cold period and the formation of inflorescences.

The decision to use ramets proved successful, as ramets of five of the plants flowering after five weeks of treatment, were also among the plants flowering after twelve weeks of treatment. Thus it can be concluded that the results were due to the duration of the treatments and that the influence of genetic variation was ruled out.

Only plants that were subjected to the colder temperature regime with continuous lighting formed inflorescences. Thus these plants were vernalized and received a long (continuous) photoperiod simultaneously. This needs further investigation, as it is normally believed that Italian type plants require vernalization, followed by longer photoperiods and warmer temperatures, for the induction of flowering.

As the only difference in the temperature regimes was the day temperature, it is also possible that the higher day temperature (23°C) may have had a negative effect on the formation of the vernalization stimulus. Hill and Pearson (1985) found that reproductive development in L. multiflorum occurred sooner in plants grown at lower temperature regimes and did not occur in those grown at day/night temperatures of 24/19°C and 30/25°C respectively. The reason why the temperature regime of 4°C at night and 23°C during the day did not induce flowering in this experiment, might be because the higher day temperature of 23°C could counteract the effect of the low temperature, so that an earlier obtained degree of induction might be lost partly, or completely (Booij & Meurs, 1992). It seems as if a certain minimum amount or critical level of vernalization is required for the induction of flowering. The colder day temperature (13°C) might have the same negative effect, only in a lesser degree, so that the critical level is reached earlier, after a shorter period of time.

As translocation of the vernalization stimulus, according to this experiment, does not seem to occur, it has important implications with respect to the grazing of seed production units containing Italian type plants. If vernalized tillers are grazed off while still in an early reproductive phase, the shoot apices, where the vernalization stimulus is perceived, are still in the basal part of the tillers and thus able to form inflorescences. If the tillers are grazed at a later stage of the reproductive phase, the inflorescences are already about half way up the tiller. This stage can easily be detected in the field by feeling a tiller between the

fingertips. The inflorescence can be felt as a little "knob" or node in the tiller. If this "node" is grazed off, the tiller will not form an inflorescence. New tillers that are formed after grazing will not flower unless the plants are vernalized again. Thus seed production units containing Italian type plants and closed down too late (at an advanced reproductive phase), are in danger of undergoing genetic shift, a change in composition, and/or a reduction in seed yield (see table 13 column 5).

2.4.4 **Examination of shoot apices**

It can be concluded from the results that the reproductive state can be determined at an early stage. Where a vernalization experiment (2.2.3) is carried out, it takes about thirty weeks to get to the stage where most of the inflorescences are formed. Although destructive, the excision of shoot apices can reduce the duration of such an experiment to ten to fifteen weeks, as was the case in this experiment.

Of every three tillers per plant that was treated, the shoot apex of one tiller was examined, while the other two tillers were left to form inflorescences later on. The fact that the plants that were reproductive according to the examination of a shoot apex, did form inflorescences later on, confirms that this method of examining the shoot apices does have value in specific instances, especially where induction and flowering are caused by different factors.

CHAPTER 3

BIOTECHNOLOGICAL TECHNIQUES

CHAPTER 3

BIOTECHNOLOGICAL TECHNIQUES

3.1 Tissue culture: meristem tip culture

3.1.1 Introduction

Meristem tip culture is mainly used as a means of vegetative propagation. It is also used to eradicate viruses from vegetatively propagated species. The technique has been of greatest interest in horticultural crops and it is believed that it could be usefully extended to pasture plants, particularly grasses and clovers. An interesting application of meristem tip culture would be in studies on the physiology of flowering. Bernier (1992) stated that classical evidence, supported by recent results, indicates that low temperatures, which promote flowering, are perceived by the shoot apex.

In grasses, such as L. multiflorum, the shoot apex develops an elongated cone during the vegetative phase with two ranks of alternating unexpanded leaf primordia, that appear as a single ridge. When these plants enter the reproductive phase, small "bulges" appear in the axils of all leaf primordia present at the time, resulting in a "double-ridge" appearance. Thus, the shoot apex in the reproductive phase can be identified by this "double ridge" when dissected out.

Vernalization experiments carried out in the glasshouse tend to be very space - and time consuming. Inflorescences usually appear weeks or months after vernalization has taken place. One is also very restricted in the number of plants that can be tested, as the utilization of glasshouses and growth chamber space are expensive and space is often limited. By using tissue culture, however, a vast number of tillers can be tested simultaneously because of the size of the samples and test tubes or petri dishes. As the shoot apices are isolated from the mother plants, one would also expect the treatment to have a much greater effect and thus be expressed in a shorter time period. Such an effect could be noted earlier than in a glasshouse trial with whole plants.

The response of in vitro shoot apices to vernalization are thus expected to be noted within a relatively short period of time. A similar experiment was carried out by Arumuganathan et al. in 1991 on L. temulentum, where plants derived from callus showed varying degrees of vernalization response. Another application was reported by Bernier in 1992. He reports that in the SDP (Short Day Plant) Pharbitis nil, flowering of apices excised from non-induced plants and grown in vitro is promoted by the addition of phloem sap from induced cotyledons to the culture medium.

In this chapter, the response of shoot apices of L. multiflorum cultivars to vernalization is investigated, as well as the viability of tissue culture techniques for determining vernalization requirements in vitro.

3.1.2 Developing the method

The explant of meristem culture may be the apical meristem or, more frequently, the apical dome with a few subjacent leaf primordia (the subapical meristematic region). Larger explants are easier to dissect and have much higher survival and growth rates than smaller explants. Actively growing shoot tips are recommended for meristem, shoot tip and bud cultures because of their strong growth potential and low virus concentration. In a morphological study of L. multiflorum, it was found that the shoot apices of vegetative tillers are situated in the base of the tiller.

3.1.2a) Problems encountered in preliminary experiments

Preliminary experiments showed areas that needed attention because they could cause problems during this study. The method of excision had to be examined, because contamination occurred, as well as difficulty in handling the meristem tips due to their size. Survival of tiller sections also depended on their size, thus a suitable size had to be found. Sterilization needed to be more efficient and the incubation temperature was not suitable for an acceptable survival rate of tiller sections.

3.1.2b) Amendments to overcome problems encountered in preliminary experiments

a) Method of excision

i) Shoot apical meristem tips of lengths 0.5-1.0mm were excised from four-week-old non-induced plants.

ii) Tiller sections, from the basal part of a non-induced tiller, containing the shoot apex, were cut from just above the roots to about 10mm long. Two to three leaf sheaths were removed, so that the shoot apex was still covered by leaf sheaths.

About 30 shoot apices were excised in each case. It was decided to use method (ii), for the following reasons:

- "open" shoot apices (i) had the biggest problem with contamination
- they are also very small and therefore very difficult to handle - everything had to be done under a microscope in the laminar flow bench. Even then some apices just disappeared into the media
- the survival rate in (i) was the lowest
- young leaves (as those used in method (ii)) or buds might be required in addition to mature leaves for photoinduction to proceed.

Table 14 Results obtained from executing the two different methods of excision of shoot apices of L. multiflorum

	Number of shoot apices surviving	**Total number of shoot apices**
Method (i)	3	30
Method (ii)	25	30

b) Size of plant material

It was noted during pilot experiments that the highest survival rate was obtained by tiller sections with a length of at least 10mm. Thus a length of 10mm was used throughout the experiment.

c) Sterilization techniques

Problems were experienced with contamination of tiller sections. Because it was believed

that tiller sections were contaminated during excision, a further period of sterilization was incorporated after excision. Various concentrations , ie. 1%, 3% and 6% of sodium hypochlorite were tested for three and five minutes. Tiller sections sterilized in a 6% solution for five minutes survived. It was, therefore, decided to use this method for a further period of sterilization.

d) Incubation of shoot apices

Shoot apices (tiller sections) were placed vertically on 5ml of MS media in test tubes and then put into a growth chamber with continuous fluorescent light illumination at a temperature of 25°C, as used by Dale (1975) for meristem tips. The tiller sections did not have an acceptable survival rate, so it was subsequently decided to use a temperature of 20°C, which proved more successful.

Explants used in this experiment were from non-induced plants of tetraploid L. multiflorum cultivars, both Italian and Westerwolds. The plants were grown in the glasshouse with a temperature ranging from 16°C to 35°C and a photoperiod of 12+ hours. At an age of four weeks, tillers were selected and trimmed to assist handling. These were then cut to about 20mm above the roots, the roots were cut off, and the sections were surface-sterilized in a sodium hypochlorite solution (6% available chlorine) for seven minutes. This was followed by six washings in sterile water. Using a dissecting microscope in the laminar flow bench, tiller sections were cut to 10mm and the outer layers of leaves removed under sterile conditions. Optical fibre illumination was used to prevent heat damage of the shoot apices. The sections were then again sterilized in a 6% sodium hypochlorite solution for five minutes and washed in sterile water. These tiller sections were then placed in test tubes on 5ml of Murashige and Skoog (MS) media (Murashige & Skoog, 1962). 0.2mg/l Kinetin was added to the medium (Dale, 1975). All components of the media, except the Thiamine, were autoclaved at 121°C for 15 minutes after adjusting the pH to 5.7. The Thiamine was poured into the media in the laminar flow bench, through a "millipore" filter.

3.1.3 **The response of shoot apices to vernalization**

From the work on ramets as discussed on Page 43, it was concluded that, for intact plants, a night/day temperature regime of 4/13°C was adequate for vernalization to occur, and that the vernalization period should be at least five weeks long. It was also speculated that higher temperatures partially break down the effect of vernalization, until a certain critical level is reached.

A vernalization period of five weeks, plus a few weeks at longer photoperiods for the formation of inflorescences, proved too long for the in vitro shoot apices involved in this experiment. After three weeks the shoot apices did not look as healthy as when the experiment was started and started dying off in the fourth week. Due to the fact that the shoot apices looked so much more vulnerable, it was decided to do this experiment using different temperatures and treatment periods.

3.1.3a) Materials and methods

i) The pilot experiment

To test the response of shoot apices to vernalization, an experiment was carried out using Italian type plants (cv. Tetrone). A pilot experiment was done in order to test the efficiency of the method, as well as to get an idea of the temperatures involved in the induction of flowering in shoot apices.

Twelve tiller sections, ie. shoot apices, of Tetrone (four months old) were tested for one week at a temperature of 0-3°C in a refrigerator. After the one week of treatment, the test tubes containing the shoot apices were taken from the refrigerator and put into a growth chamber with continuous lighting and a temperature setting of 16°C. After two weeks in the growth chamber, the shoot apices were dissected out to see whether they had been vernalized (reproductive phase) or not (vegetative phase). The reproductive and vegetative shoot apices were counted and noted.

ii) The first experiment

For this experiment, thirty six tiller sections of six weeks old of Tetrone and Billion, prepared as for the pilot experiment, were tested for three treatment periods, 0, 1 and 2 weeks, at a temperature of 0-3°C in a refrigerator. There were 12 replications with six test tubes in each replication. One test tube, containing one tiller section, was an experimental unit. After one and two weeks of treatment, the particular test tubes were taken from the refrigerator and put into a growth chamber with continuous fluorescent lighting and a temperature setting of 20°C. The control, with no treatment, was kept in the growth chamber for the duration of the experiment. Every two weeks, the tiller sections were placed on fresh media and returned to the growth chamber. Three weeks after each cold treatment, the shoot apices were dissected out to see whether they had been vernalized or not.

iii) The second experiment

Thirty six tiller sections, six weeks old, of both Tetrone and Billion were again treated for two time periods, 1, and 2 weeks, at a temperature of 0-3°C in the refrigerator. A control ("0-week treatment") was also included in this experiment. There were twelve replications. The control was kept in the growth chamber for three weeks, the "1-week treatment" for two weeks after the cold treatment, and the "2-week treatment" for one week after the cold treatment. Thus, the duration of the experiment for each treatment was three weeks. The temperature in the growth chamber was set for 13°C and the fluorescent lighting was continuous. After the experiment was completed, the shoot apices were dissected out and examined for the reproductive and vegetative phase.

3.1.3b) Results

i) The pilot experiment

Of the twelve shoot apices examined, only three were in the reproductive phase. The results are presented in table 15.

Table 15 The response of Tetrone ryegrass shoot apices, two weeks after an induction treatment of one week at 0-3°C

PLANT NUMBER	REPRODUCTIVE (R) or VEGETATIVE (V)
1	V
2	V
3	R
4	R
5	V
6	V
7	V
8	V
9	R
10	V
11	V
12	V

ii) The first experiment

No positive results were obtained from the seventy two tiller sections examined - all of them were still in the vegetative phase.

iii) The second experiment

Again, no positive results were obtained from the seventy two shoot apices examined. They were all in the vegetative phase. The position of the shoot apex, as well as the shoot apex itself in L. multiflorum, can be seen in figure 5.

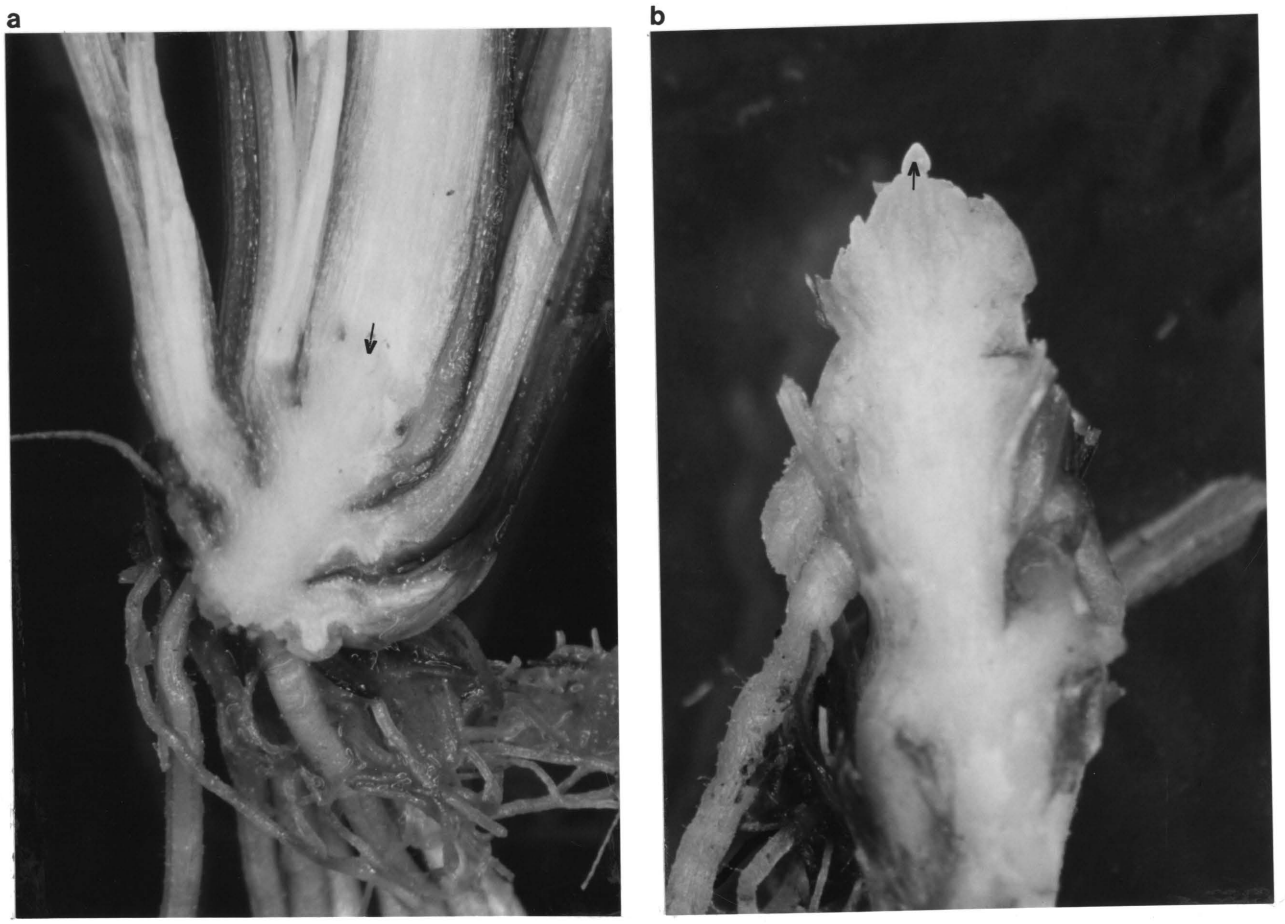

Figure 5 The position of the terminal bud of the main shoot apex (a) and the exposed terminal bud (b)

3.1.3c) Discussion

i) The pilot experiment

Because some positive results, although minimal, were obtained, it was decided to repeat the experiment on a bigger scale, including a control. As only three tillers became reproductive, it was believed that changing the period of treatment or the period in the continuous lighting in the growth chamber, more positive results could be obtained. The temperature in the refrigerator was believed to be cold enough for the induction of flowering and was consequently not changed.

ii) The first experiment

Billion was not expected to form any inflorescences as a result of the treatments. If inflorescences were formed, it would have been as a result of the continuous lighting. There are various possible explanations for the unsuccessful treatment of Tetrone. The three-week period in the growth chamber might have been too long at such a high temperature (for ryegrass). From earlier experiments (chapter 2) it could be concluded that ryegrass grows more vigorously at lower temperatures, or should at least have a very low night temperature. Here the temperature was set at 20°C for three weeks after each treatment. The tiller sections could have been stressed due to this fact, and growth might have been slowed down. Because it was still believed that the treatment temperature was cold enough for vernalization to occur, it was concluded that the conditions after treatment was unfavourable for the formation of inflorescences, even if vernalization initially did occur. Due to the rather high temperature and long period of time (three weeks) in the growth chamber, it was believed that de-vernalization could occur because of the short treatment periods which meant that vernalization probably could not reach the critical level needed to avoid de-vernalization.

Areas that needed to be tested further, were the period of time spent by tiller sections in the growth chamber, as well as the temperature in the growth chamber. The treatment periods could not be changed, because the possibility was still there that the tiller sections could die if the experiment carried on for too long.

iii) The second experiment

This led to two possible conclusions. Firstly, although a temperature of 13°C was successful as a maximum or day temperature in plants, it was either too cold for the shoot apices, or should not have been a continuous temperature, but part of a night/day temperature regime, to allow the formation of inflorescences. Secondly, a more probable conclusion is that tillers at an age of six weeks are still in a juvenile phase and not yet receptive to vernalization.

Many grass species must go through a juvenile phase before becoming receptive to flower induction stimuli (Cooper & Calder, 1964, sited in Aamlid, 1992). The fact that L. multiflorum could not be vernalized in the seed stage (chapter 2), suggests that this species must go through a juvenile phase. Therefore each individual tiller will have its own juvenile phase, flower induction requirement and vernalization must be perceived by each individual tiller (Aamlid, 1992).

It is essential for further work to be done in this respect, because if L. multiflorum does have a juvenile phase and translocation of the vernalization stimulus can be ruled out, this will then definitely have important implications for the grazing of seed production units containing Italian type plants.

3.2 Electrophoresis

3.2.1 Introduction

Electrophoresis is widely used to examine the composition and dynamics of stands, as well as competitiveness within cultivar mixtures. An advantage of this technique is that conventional stands can be examined in situ, or as harvested material. Thus, it is almost non-destructive.

Gentner used fluorescence tests to distinguish L. multiflorum and L. perenne, but it has been found that seedlings of L. perenne were also fluorescing (Gardiner et al., 1986). A search for alternative methods to distinguish between these two species, has lead to various studies on the use of electrophoresis. The different protein banding patterns that were obtained in individual cultivars of L. multiflorum, indicated the potential use of these methods to distinguish cultivars and species.

Seed proteins are more widely used, because soil and the age of the seed do not affect the protein composition. Protein bands are supposed to be independent of environment, year or generation of seed production, and are not affected by endophytes (Gardiner & Forde, 1987). One big advantage here is that unimbibed seed is at the same physiological stage. However, where more cultivars are involved, it is better to use vegetative plant material, because the bigger morphological differentiation between cultivars can result in more distinctive protein bands (Nakamura, 1979).

The basic principle of electrophoresis is simple. Two electrodes, with a potential gradient, are placed in a solution. Cations and anions will then move to the cathode and anode respectively. The movement of the ions is determined by the electric current and the nett charge on the ions, and is slowed down by friction, due to the size and shape of the ions, as well as the viscosity of the solution. Ions differing in size, shape or charge can be separated in principle (Andrews, 1986).

Although the SDS PAGE technique is effective in identifying grass seed cultivars, it is not as effective for the identification of plant material (Murphy et al., 1990). Leaf protein

composition can vary with age and environmental conditions, like temperature, photoperiod, or nutrition. In this study, however, the effect of the vernalization stimulus on the plant was examined by using the SDS PAGE technique. Plant material, vernalized and unvernalized, was examined in order to see if temperature and photoperiod affects the protein banding pattern of leaves of L. multiflorum. It was postulated that a "shock-protein" might be produced in reaction to vernalization, which might result in a change in the protein banding pattern. Gardiner and Forde (1987) also used SDS PAGE for the identification of grass seed proteins.

3.2.2 Materials and methods

"Grab samples" of leaves of the Italian cultivar Tetrone, and the Westerwolds cultivar, Billion, were collected from different temperature treatments applied to intact plants treated as described in chapter two. Protein extractions were made from crushed leaves, frozen in liquid nitrogen, using a mortar and pestle. A few drops of an extraction buffer (see appendix) were added. The extracts were poured into Eppendorf tubes and centrifuged for fifteen minutes at 4-9°C and a speed of 15 000 revolutions per minute (15 600 x g). The supernatant was poured off into clean Eppendorf tubes and frozen until used.

Protein extracts were electrophoresed on acrylamide gels which were 16cm long, 14cm wide and 0.15cm thick. Gels were made up as described in the appendix. Equal parts of the disruption buffer and the protein extracts were mixed. Twenty microlitres of the suspension were introduced to the bottom of each well in the gel using a Hamilton microlitre syringe. Twenty microlitres of a standard molecular weight marker were loaded into the two outside wells in the gel. Two gels were run simultaneously at fourty milliamperes for four hours in the refrigerator, using a Consort E455 Microcomputer electrophoresis power supply, until the front was two centimetres from the end of the gel. Gels were then fixed and stained (see appendix). The protein banding patterns were compared visually, and the results were recorded photographically.

3.2.3 Results

The samples used were from the experiment on the vernalization of genets as described in chapter two. Treatments that were to be compared were run on the same gel, so that the conditions were the same and any difference would mainly be due to treatment.

In gel #1 (figure 6a), samples of Tetrone (Italian) and Billion (Westerwolds) were run. All the samples were from material treated at a night/day temperature regime of 10/23°C, which was thought to be non-inductive, with two photoperiods, nine hours and fourteen hours. The protein bands were compared, using the molecular weight markers merely to divide the bands into zones. In both Billion and Tetrone, the same bands could be distinguished. The only difference was that the bands of the samples in the long photoperiods (14 hours) were weaker than those from the short photoperiods (9 hours), although the volume of the samples were the same.

In gel #2 (figure 6b), the different temperature treatments of Tetrone, all with a fourteen hour photoperiod, were compared. No differences could be noted, except that the two lanes from samples with a colder day temperature, ie. 13°C, had thicker protein bands in zone A. It is possible that either more bands were formed, close to each other in this lane, or that a bigger quantity of that particular protein was formed, as a result of the colder temperature regimes.

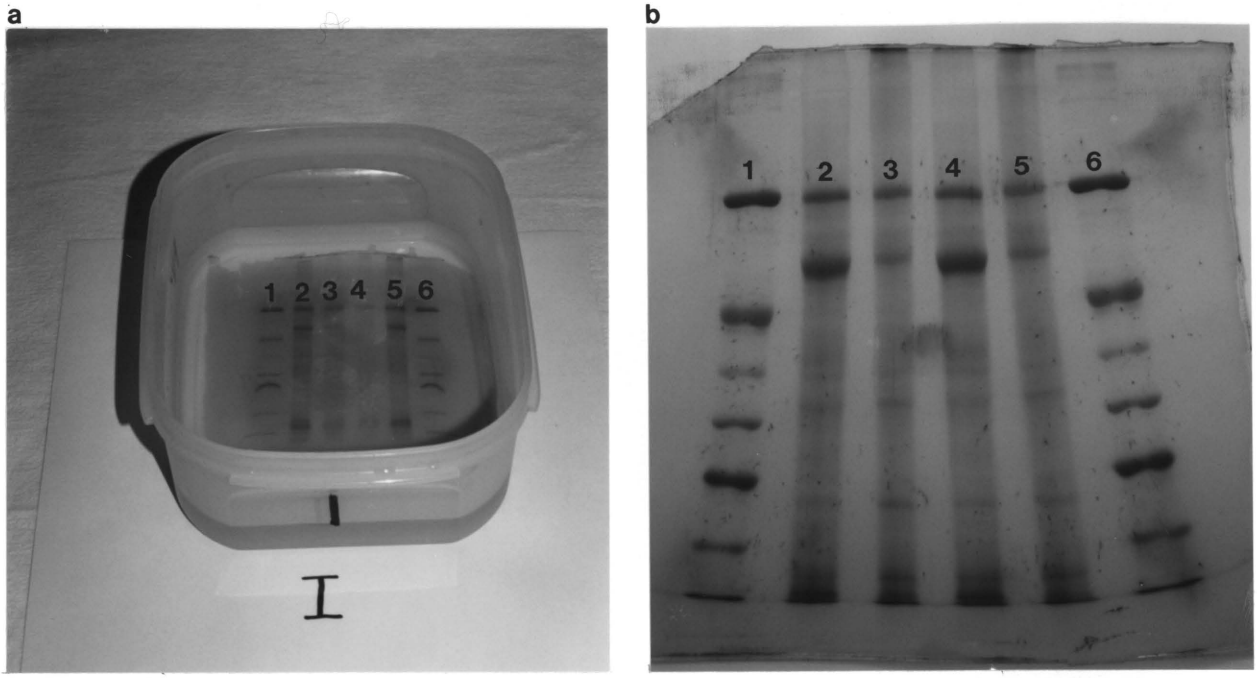

Figure 6 Gels from SDS PAGE performed on L. multiflorum vegetative plant material. The lanes represent, from left to right **(a, gel #1)**: molecular weight marker, Billion (9 hours), Billion (14 hours), Tetrone (14 hours), Tetrone (9 hours), molecular weight marker and **(b, gel #2)**: molecular weight marker, Tetrone 10/13°C, Tetrone 10/23°C, Tetrone 4/13°C, Tetrone 4/23°C, molecular weight marker

In gel #3 (figure 7a), the two colder temperature regimes of Tetrone (10/13°C and 4/13°C), with long photoperiods, were compared, together with Billion, the control. The bands in lane 2 (Billion, short photoperiods) were stronger than those in the other lanes. In lane 5 (Tetrone 4/13°C), a band appeared in zone C that did not develop in the other lanes. This was a sample taken from the coldest temperature treatment.

In gel #4 (figure 7b), the two warmer temperature regimes of Tetrone (4/23°C and 10/23°C, long photoperiods) were compared, together with Billion, the control. The two Billion samples (lanes 4 and 5) had stronger bands than the two Tetrone samples. No new bands could be detected. In this gel, all the samples came from a day temperature of 23°C. Lanes 2 and 3 were from Tetrone and lanes 4 and 5 from Billion. Lane 5 was the only sample from a short photoperiod and had the strongest bands.

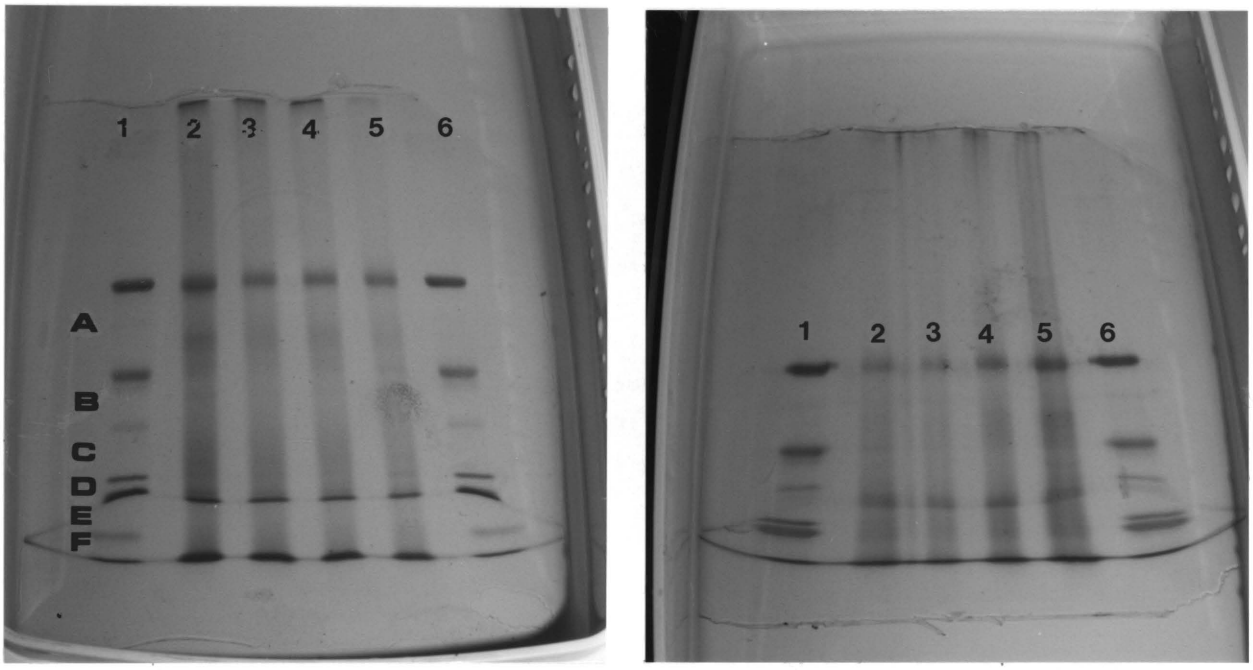

Figure 7 Gels from SDS PAGE performed on L. multiflorum vegetative plant material. The lanes represent, from left to right **(a, gel #3)**: molecular weight marker, Billion 10/23°C (9 hours), Tetrone 10/13°C (14 hours), Billion 10/23°C (14 hours), Tetrone 4/13°C (14 hours), molecular weight marker and **(b, gel #4)**: molecular weight marker, Tetrone 4/23°C (14 hours), Tetrone 10/23°C (14 hours), Billion 10/23°C (14 hours), Billion 10/23°C (9 hours), molecular weight marker

3.2.4 Discussion

From the results above, it is evident that samples from plants grown at shorter photoperiods had stronger protein bands, which could indicate that more of those particular proteins were formed under those conditions.

The only differences that could be detected between Tetrone and Billion were where the different temperature treatments were compared in gel #3. Here a new band appeared in the sample taken from the coldest Tetrone temperature treatment (4/13°C). Within Tetrone, the colder temperature regimes resulted in thicker bands, which indicate either that different (new) protein bands had developed, or that more of that particular proteins were formed.

Thus, it can be concluded that shorter photoperiods and colder temperature regimes do result in different protein banding patterns. Because the temperature treatments described in chapter two were unsuccessful for vernalization, it can not be assumed that vernalized or induced material can be detected using PAGE. This experiment merely showed that cold temperatures and short photoperiods result in different protein banding patterns. Therefore this aspect needs further investigation.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

This study was undertaken for two reasons, namely first to clarify the difference between Westerwolds and Italian type plants of L. multiflorum with respect to their inductive requirements for flowering and secondly to determine why Italian-type plants fail to become vernalized in certain climatic regions of South Africa, aspects on which no work has been undertaken in South Africa. For logistic reasons the study was conducted with two cultivars only, namely Billion, a true Westerwolds type and Tetrone, an Italian type. As pointed out in earlier discussions, many areas require further investigation.

Failure to achieve vernalization by treatment of seed, led to the conclusion that L. multiflorum can only be vernalized at plant stage and not as seed. This corresponds with the statement of Wareing and Phillips (1978) that, generally, species which can only be vernalized at the plant stage have an obligate chilling requirement. This means that cultivars containing Italian type plants should be planted in autumn to ensure vernalization of plants in winter. Grabe (*1991, pers. comm.), however, succeeded in vernalizing seed of the perennial ryegrass L. perenne. This might indicate that L. perenne does not need to pass the juvenile phase to become receptive to flower induction stimuli.

For the vernalization of plants, the results showed that a temperature regime of 4°C at night and 13°C during the day, for the duration of at least five weeks, is possibly adequate, though Wareing and Phillips (1978) reported that temperatures from -1°C to 9°C are effective for the vernalization of the majority of species. There was also found to be a correlation, or rather a dependency, between the duration of the cold period and the formation of inflorescences. Thus, the "dosage" of vernalization is important. It had been believed, however, that Italian type plants require vernalization, followed by longer photoperiods and warmer temperatures, for the induction of flowering. Wilkins (1984) reported that a vernalization requirement for flowering is often linked with a particular photoperiodic requirement, the most common being the need for cold followed by long

* Grabe, Professor D.F., Oregon State University, Oregon, U.S.A.

photoperiods.

Further investigation is important, because it was found that plants which moved into the reproductive phase received a cold treatment and continuous lighting simultaneously.

Contrary to the results of the experiment carried out by Purvis (1948) and reported by Schwabe (1954), where it was concluded that translocation of the vernalization stimulus did occur in rye plants, it was evident from this study that translocation of the vernalization stimulus does not seem to occur in the cultivar used (Tetrone). This has important implications with respect to the grazing of seed production units containing Italian type plants, an aspect that will be discussed later in this chapter.

Bernier et al. (1981, vol. II) reported that when a plant enters the reproductive phase, it results in the shoot apex having a "double-ridge" appearance. The method of examining the shoot apices for the reproductive phase soon after vernalization, although destructive, proved to have value in this type of study.

The response of shoot apices (in vitro) to vernalization was only partly positive. The shoot apices could only be vernalized for one to two weeks, however, due to the vulnerability of the excised tips. They started dying off when treated for more than two weeks. The partly positive results might have been due to the fact that the temperature regime after vernalization was not inductive for the formation of inflorescences in intact plants. A more probable conclusion was that L. multiflorum must go through a juvenile phase before becoming receptive to flower induction stimuli, as some grass species do, according to Cooper and Calder in 1964 (sited in Aamlid, 1992). The fact that seed of L. multiflorum could not be vernalized in this study, might be proof of this need to pass the juvenile phase.

As mentioned earlier, translocation of the vernalization stimulus in L. multiflorum probably does not occur. In light of the possibility of the need to pass the juvenile phase mentioned above, this then implies that each individual tiller must be vernalized.

Wilkins reported in 1984 that new proteins which appeared in winter wheat during a cold treatment resembled those that occurred in a spring cultivar after vernalization. The PAGE performed on the samples in this study indicates that cold temperatures and short photoperiods possibly change the protein banding patterns in L. multiflorum. It can only be concluded that larger quantities of existing proteins or new protein bands are formed. It can not be assumed that vernalized material can be detected, only that cold temperatures and short photoperiods result in different protein banding patterns. This aspect therefore needs further investigation. New proteins might contain a certain vernalization substance that could lead to the isolation and transfer of a substance that would induce flowering in L. multiflorum.

This study has important implications for grazing and pasture management of seed production units of L. multiflorum. Thus cultivars containing Italian type plants should be planted in autumn, well before the onset of winter, in order to have as many tillers as possible available for vernalization - this to ensure the optimum number of reproductive tillers and therefore maximum seed yields. According to this study, plants should be past the juvenile phase, ie. older than six weeks, before being subjected to vernalization. If pastures containing Italian type plants are not utilized as seed production units, but for grazing, these pastures can be established in spring. Italian type plants will then remain vegetative for a year, until after the next winter.

It is also believed that certain climatic regions might either not be cold enough, or not have cold temperatures for a long enough period of time. It seems as if there is a critical minimum level of vernalization needed to avoid "de-vernalization", a term used by Bernier et al. (1981, vol. I). It might be that high day temperatures break down an amount of the vernalization caused by low night temperatures, until a certain minimum level of vernalization is reached, after which de-vernalization does not occur.

It is evident, from the morphological study, that the growing tip, ie. shoot apex, is situated under the soil surface. Thus it can not be grazed off in the vegetative or early reproductive phase, but in the later reproductive phase, when the young inflorescence is elongated within the sheath of the tiller and can be detected as a little knob, it is situated

well above the soil surface and can be grazed off.

An early spring can cause early induction of flowering in ryegrass pastures and reproductive tillers, with the young inflorescences already above the soil surface, could be grazed off before the close down date of 15 October. As translocation of the vernalization stimulus does not occur, seed production of Italian type plants would be drastically reduced unless it goes through the following winter. If each pasture could be "tested" for the reproductive state by feeling the tillers, grazing animals could be removed as soon as the inflorescences (knobs within the tillers) could be detected. If too many inflorescences have already been grazed off, grazing could continue and seed production forsaken. This way genetic stability and the composition of mixed cultivars could be maintained. It is consequently believed that the close down date for seed production units should be revised or substituted with a test in each particular pasture.

The low frequency of ryegrass plants entering the reproductive phase during these experiments could perhaps be attributed to the fact that ryegrass was primarily selected for maximum forage production which is normally negatively correlated to seed production.

Contrary to the normally expected transition from the vegetative to the reproductive phase due to vernalization, it seems that other stress factors that disturb the normal plant rhythms such as, in these experiments, cutting and continuous light, could also induce this transition. Should such factors be able to induce these changes independently, their importance should not be underestimated.

It is therefore important that further research on the induction of flowering should also take stress factors other than vernalization into account. This study can be seen as a starting point and further work, especially specific case studies, is essential for this problem to be solved, as many questions on the induction of flowering in L. multiflorum still can not be answered with certainty.

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APPENDIX

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

1.1 Extraction buffer (Swain, 1989)

4 g Polyclar

5.13 g sucrose

0.009 g EDTA

0.008 g dithiothrietol

0.009 g ascorbic acid

0.05 g Bovine Serum Albumin

0.013 g NAD[1]

0.012 g NADP[2]

0.0026 g pyridoxal 5^1-phosphate

50 ml distilled water

Adjust to pH 6.7 with 1M Tris and add 0.33 ml B-mercaptoethanol.

[1]Nicotinamide adenine dinucleotide

[2]Nicotinamide adenine dinucleotide phosphate

1.2 Reagents

1.2.1 30% Acrylamide (Salm, 1990)

30 g acrylamide

0.8 g N,N^1-methylene bisacrylamide

100 ml distilled water

This was filtered through Whatman number one filter paper and stored in a dark bottle at 4°C.

1.2.2 10% SDS (Sodium dodecyl sulphate) (Salm, 1990)

10 g SDS was added to 100 ml distilled water.

1.2.3 1.5M Tris pH 8.8 (Salm,1990)

18.2 g of Tris (Boehringer Mannheim) were dissolved in 100 ml distilled water and the pH adjusted to 8.8 by using 1M HCl.

1.2.4 0.5M Tris pH 6.8 (Salm, 1990)

7.9 g of Tris-HCl (Boehringer Mannheim) were dissolved in
100 ml distilled water and the pH adjusted to 6.8 using
1M HCl.

1.2.5 10% Ammonium persulphate (Salm,1990)

0.1 g of ammonium persulphate was added to 1 ml of distilled water.

1.2.6 Electrode buffer (Salm, 1990)

14.4 g glycine
16.5 ml 1.5M Tris pH 8.8
10 ml 10% SDS
956.6 ml distilled water

1.2.7 Disruption buffer (Salm, 1990)

0.1M Tris-HCl pH 6.8
2.5% (w/v) SDS
5% (v/v) mercaptoethanol
5% (v/v) glycerol
Bromophenol Blue (0.5 mg/9 ml solution)

1.3 Gel preparation

1.3.1 5% (w/v) Stacking gel (Salm, 1990)

The stacking gel was prepared by mixing the following amounts of the stock solutions in a 50 ml glass Erlenmeyer flask:
1.8 ml 30% acrylamide stock
2.5 ml 0.5M Tris pH 6.8

0.1 ml 10% SDS

0.2 ml 10% ammonium persulphate

5.4 ml distilled water

12 µl N,N,N^1,N^1-tetramethylethylene diamine (TEMED)

1.3.2 10% (w/v) Resolving gel (Salm, 1990)

The 10% resolving gel was prepared by mixing the following amounts of the stock solutions:

6.7 ml 30% acrylamide stock

5 ml 1.5M Tris pH 8.8

0.2 ml 10% SDS

0.4 ml ammonium persulphate

7.7 ml distilled water

15 µl TEMED

1.3.3 Procedure (Salm, 1990)

For the resolving gel, the mixture was stirred, with the TEMED added while stirring. The mixture was poured into alcohol-washed glass plate assemblies with 1.5 mm spacers and overlayed with water-saturated butanol. Polymerisation was allowed for an hour at 4°C. The stacking gel mixture was then stirred, with the concurrent addition of the TEMED and poured into the remaining space in the glass plates. Ten-place perspex combs were inserted and polymerisation allowed at 4°C for another hour. After polymerisation, the combs were removed and the glass plates fixed to the electrophoresis equipment. The wells were filled with electrode buffer and left until the gels were run.

1.4 Gel fixation (Giulian et al., 1984)

1.4.1 Fixative 1

40 g TCA (trichloro-acetic acid)

make up to 200 ml with distilled water

1.4.2 Fixative 2

200 ml ethanol

50 ml acetic acid

1.25 g SDS

make up to 500 ml with distilled water

1.4.3 Procedure

After electrophoresis was completed, gels were fixed by shaking them gently for thirty minutes, first in fixative 1, then in fixative 2 for another thirty minutes.

1.5 Gel staining (Giulian et al., 1984)

1.5.1 Stain

40 ml ethanol

10 ml acetic acid

12.5 ml 1% solution Coomassie Blue R-250

make up to 100 ml with distilled water

1.5.2 Destain

200 ml ethanol

50 ml acetic acid

make up to 500 ml with distilled water

1.5.3 Procedure

Gels were stained by shaking them gently in the stain for three hours, and destained until the banding pattern could be clearly distinguished from the rest of the gel.

Gels were stored in a solution containing 7% acetic acid and 5% methanol in the refrigerator.

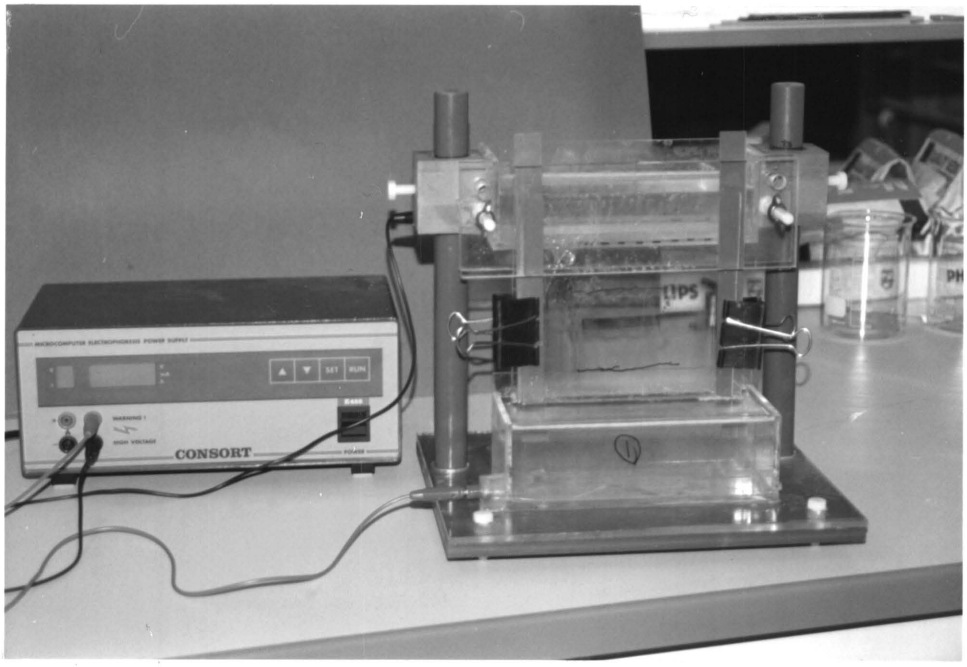

Figure 8 Equipment similar to the vertical slab gel apparatus of Studier (1973) was used for the SDS PAGE (taken from Andrews, 1986)

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