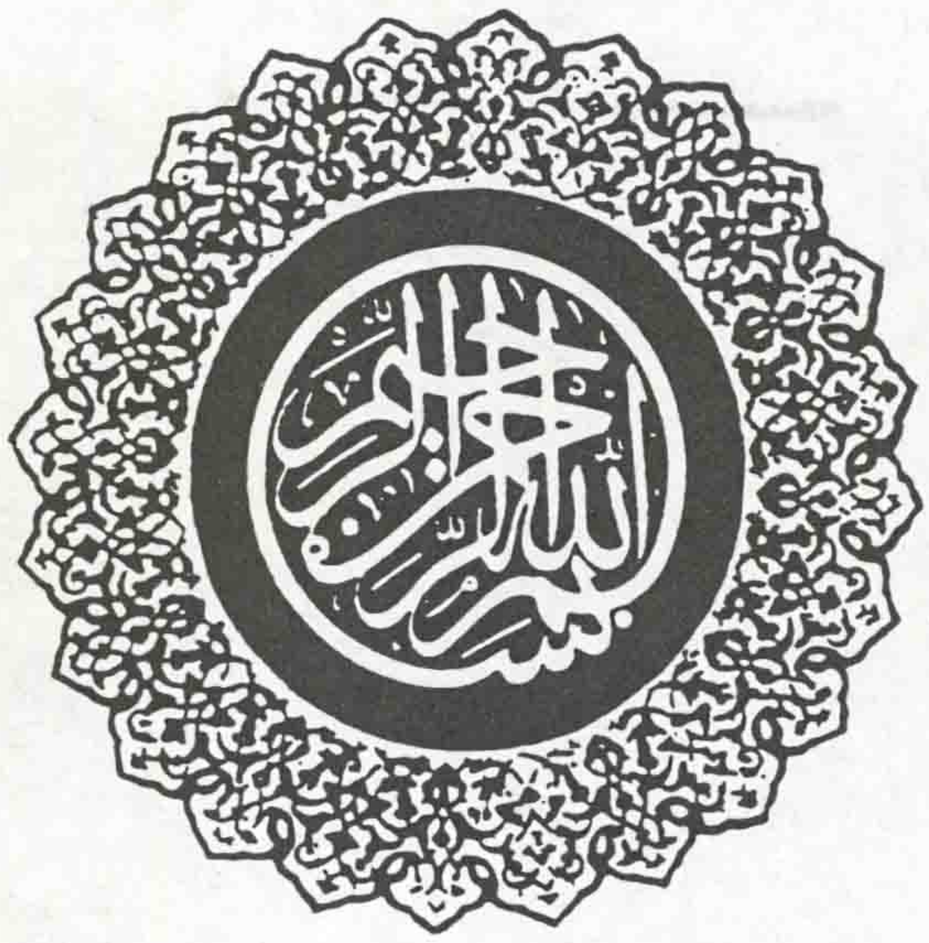

IN

THE NAME OF ALLAH,

THE BENEFICENT, THE MERCIFUL

THE UNIVERSITY
of LIVERPOOL

ANTHER CULTURE
IN
INTERGENERIC HYBRIDS OF FORAGE GRASSES

by

Abbas Ghamari Zare

A thesis submitted to the University of Liverpool in fulfilment of the requirement for the degree of

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To

My son, my wife and my parents

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ABSTRACT

The two genera *Festuca* and *Lolium* include most of the perennial grass species that are sown for forage in temperate areas. The combination of *Lolium multiflorum* and *Festuca arundinacea* in a single genotype, has been claimed to offer the best mixture of forage characters. *Lolium* generally provides high quality nutritious forage and *Festuca* provides better adaptation to environmental stresses such as drought and cold.

More than 2,300 androgenic plantlets which displayed a wide range of morphological, and growth characteristics were produced from a *Lolium multiflorum* × *Festuca arundinacea* pentaploid hybrid (2n = 5x = 35).

In the screening for cold tolerance, androgenic plants and their parents as controls were exposed to freezing temperatures ranging between -2°C to -14°C. Some androgenic plants displayed freezing tolerance ranging beyond the extremes of the cold tolerant *Festuca* species.

In the screening for drought resistance, tillers of androgenic plants and their parents as controls were exposed to continuous drought for 110 days under a rain-out shelter in a field and in a separate experiment, 77 days in brick bins in a glasshouse. Androgenic plants were identified with superior growth under drought conditions than drought tolerant *F. arundinacea* and with higher tiller production than drought susceptible *L. multiflorum*.

The genetic composition of androgenic plants was determined by isozyme and cytological studies which included genomic *in situ* hybridization (GISH). The parent hybrid was labelled at the PGI/2 locus by five different homoeoalleles each located on a different chromosome in a homoeologous chromosome set in the hybrid. By comparing PGI/2 allele segregation, levels of preferential chromosome pairing in the hybrid and of genetic variation within the polyhaploid population could be determined. There was preferential chromosome pairing in the hybrid between the homologous *Lolium* chromosomes so that nearly all gametes carried a complete or near complete *Lolium* genome.

Interspecific and intergeneric recombination in the pentaploid hybrid was confirmed by GISH on mitotic chromosome preparations of two stress tolerant androgenic genotypes. Both plants contained *Lolium*, and *Festuca* chromosomes, and the chromosomes of *F. arundinacea* could be distinguished as *F. pratensis* or *F. glaucescens* (the progenitors of the hexaploid *Festuca* species), by GISH. Recombinants were found between chromosomes of *L. multiflorum*, *F. pratensis*, and *F. glaucescens*. These included a chromosome segment of *F. pratensis* known to carry genes for drought tolerance.

Androgenesis of *Festulolium* hybrids revealed unique gene combinations and extremes of variation not found in the parent species. Selected gene combinations should be suitable for commercial exploitation once they are made homozygous by chromosome doubling and are fertile and chromosome stability is achieved.

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Abbreviations and symbols

°C	degrees Celsius
µg	micro gram
µl	micro litter
µmol m^{-2} s^{-1}	micro moles per square metre per second
%	per cent
ACD plant	anther culture derived plant
bp	base pair
chr	chromosome
cm	centimetre
cm^2	square centimetre
CMD	cell membrane damage
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
e.g.	for example
Env	environment
Fa	*Festuca arundinacea*
FAO	Food and Agricultural Organization of the United Nations
Festulolium	*Lolium multiflorum* × *Festuca arundinacea* pentaploid hybrid
Fg	*Festuca glaucescens*
Fp	*Festuca pratensis*
g	gram

Geno	genotype
GISH	genomic *in situ* hybridization
gl^{-1}	gram per litre
GLM	General Linear Models Procedure
H_0	null hypothesis
h	hours
IGER	The Institute of Grassland and Environmental Research
in^2	square inch
IRRI	The International Rice Research Institute
kb	kilo base
kg	kilogram
lbs	pounds (1 pound = 453g)
Lm	*Lolium multiflorum*
Lm/Fa	*Lolium multiflorum* × *Festuca arundinacea* pentaploid hybrid
LSD	least significant differences
LT_{50}	the lethal temperature for 50% kill
m	metre
M	Molar
mA	milli Ampere
MAS	marker assisted selection
mg	milligram
mgd^{-1}	milligram per day
min	minutes
ml	millilitre
mm	millimetre
mm^2	square millimetre
N	Normal
PEG	polyethylene glycol
pers. comm.	personal communication

PGI/2	phosphoglucoisomerase
PGR	plant growth rate
P_{H0}	the probability of the null hypothesis
PPFD	photosynthetic photon flux density
ppm	part per million
Pr	probability
PRIN	principal component analysis
QTL	Quantitative Trail Loci
r	correlation coefficients
Rep	replication
RFLP	Restriction Fragment Length Polymorphism
RWC	relative water content
S.W.	South-West
SAS	The Statistical Analysis System
SS	survival score
TGP	total genomic DNA probe
USA	The United Stated of America
v/v	volume per volume
W/m^2	watt per square metre

CHAPTER ONE

GENERAL INTRODUCTION

1. 1. Importance of grass and grassland

Our life is so inextricably involved with that of the grasses which grace our fields that the study of grassland is both fascinating and intriguing to all who possess an inquiring mind, be they born and bred in towns or sons of the soil. What is more, the management of the grass sward for farming, sport or leisure offers a real challenge to these rival developments in understanding the technical advances in the realms of botany, chemistry, engineering and economics. The significance of grasses in the life of man was recognised in earliest times. However the distractions of modern life in the great cities and the speed with which man now passes through the countryside have caused him to underestimate its importance (Moore, 1964).

Unlike other crops grown for their grain or vegetative parts to benefit man directly, grass and grassland products are almost entirely for the feeding of ruminant animals; the interaction of the sward and the animal thus adds an extra dimension to investigations of the productivity of grassland. The immediate benefit of forage grasses is mainly throughout their conversion to meat, milk and fibre, but they also offer high value both by their soil-ameliorating effects in primitive shifting cultivation and as break crops in the sophisticated cropping schedules of mechanized agriculture (Sneep, Murty and Utz, 1979). In some countries, for example the UK, Australia and New Zealand, animal production from grassland makes a greater contribution to agricultural production than does any other crop (Jones and Lazenby, 1988).

Grassland based products, include meat, dairy products and wool, and comprise an important part of the human diet and apparel. Much of world production of meat, dairy products and wool remains essentially pasture based, although some 60% of beef production and about 75% of dairy production also relies, to some extent, on supplementary feeding of grain and concentrates, especially on North America, Japan, Europe and the Commonwealth of Independent States (Graeme *et al*., 1993).

According to the FAO data (1992), the area of permanent meadows or of pastures during the last two decades increased by about 275 million hectares, or 8.8% (Table 1.1). However the increase of livestock and population pressure on the environment show that its use has been consistent during those decades (Table 1.2, FAO, 1992).

Table 1.1. World permanent meadows and pastures

	Area in '000 ha		
	1970	1980	1990
World	3216875	3116685	3402077
Developed world	1279584	1269728	1235144
Developing world	1847291	1846959	2166933

Table 1.2. The world livestock and population on the environment

	Ratio of permanent pasture (ha) to sheep (head)			Ratio of arable and permanent crops (ha) to total population		
	1970	1980	1990	1970	1980	1990
World	2.94	2.79	2.80	0.38	0.32	0.27
Developed world	3.04	3.66	2.20	0.63	0.58	0.54
Developing world	4.76	3.90	3.26	0.28	0.23	0.18

The population, particularly in the poor nations and developing countries is increasing but the land area is constant and the demand for food is increasing every day. To meet these demands there is no alternative except to increase the yield per land unit through improved grassland management and potential yield of forage crops.

1. 2. Distribution of the grasses

The *Graminae* is one of the largest families of flowering plants in number of genera and species. Of an estimated total 12500 genera of flowering plants, 600 or 4.8% are grasses, and of an estimated total of 225000 species, 7500 or 3.3 % are grasses (Good, 1953). Geographically the grasses are ubiquitous, and the grass family is truly cosmopolitan. Grasses occur in all continents, including Antarctica. Indeed it would be hard to find any area of substantial size where grasses are absent, except those which are too barren or cold to support the growth of any higher plants (Vester, 1940).

Grasses are present in almost all types of vegetation, not only in the prairies and steppes where they are dominant, but also in temperate and tropical forests, deserts, and swamps. Any account of distribution of grasses which is confined to consideration of only grassland swards would fail to reveal some of the most significant features of the family, for the grasses as a family are outstanding in their ability to adapt to diverse ecological conditions (Bartley, 1966). However grasses occur most typically in the interior of the great continental land masses, ranging Northward to about 55° N in Asia and North America, and southward to 40° S in South America. Treeless prairies, steppes and pampas predominate in the temperate regions, and are replaced by savannahs, with varying proportions of trees and shrubs, in the tropics and sub-tropics (Bartley, 1966). In all, Shantz (1954) estimated the grasslands to account for almost one-quarter of the world's cover of vegetation.

1. 3. Importance of grass and grassland in Iran

Grassland is a particularly important crop in the Middle East. Iran which is the largest country in the Middle East with an area of 1.65 million km^2 is located between latitude 26° to 38° N and longitude 44° to 63° E. Because of this wide range in latitude and longitude, Iran has a diverse type of climate, vegetation and

biological productivity. While most of the Caspian shore plain is below sea level, the Damavand mountain is 5671 metres high. Unfortunately the distribution of the rainfall is not uniform. Iran's climate is characterised by aridity with more than 30% of the country receiving less than 100 mm of precipitation annually, while in contrast much of the Gilan province in the Caspian area receives 1000 - 2000 mm annually. In general, winter is the rainy season and summer is the driest season all over Iran. Furthermore winter is very cold especially on the mountainous parts of Iran which Koocheki (1993) regards as the most important source of food for nomads.

In Iran, as with most arid land ranges of the world, grasses are the most important of the vegetation available to grazing animals (Jafari, 1990). There are roughly 90 million hectares of land ranges with an annual dry matter production of about ten million tons (FAO, 1971; Jahad, 1988 and Koocheki, 1993): Of this, 14 million hectares is good herbaceous pasture (first class summer resort pasture) of which the annual dry herbage production is about 580 kg/ha, 60 million hectares shrub pasture (winter resort pasture, of medium class) with an annual dry herbage production of about 184 kg/ha and 16 million hectares of poor pasture (marginal area of desert) with an annual dry herbage production of about 50 kg/ha (Jangal and Marta, 1993; Frouzesh, 1995b).

For the best utilization of these pastures only half of the pasture should be used for grazing and the other half should be left for seed production (Moniee and Azez, 1980). Since the pasture is not usually in private ownership overgrazing and ill management have demolished the land ranges. However in the last decade by privatization, replanting and introduction of new genotypes, fertilization, changing poor arable land to pasture, and a better management system, the annual dry herbage matter has been increased to about 15 million tons (Fallah, 1996). Annual dry herbage production may be improved further to 30 million tons (Jehad, 1988) by improving land range management and introducing improved new resistant genotypes that combine environmental stress resistance with high productivity.

Breeding for resistance or tolerance to environmental stress is essential to improve the grasslands and increase the living standards of its farmers. The need for improved grassland is a principle that can be applied to all areas where grass is a dominant species.

1. 4. Grass breeding

When farmers began harvesting the first domesticated plants in about 8000 B.C. the earth's population was estimated to be four million people. Today, the world population exceeds five billion (Kung, 1993) and, is expected of be nearly eleven billion between 2030 to 2050 (Vasil, 1995). In anticipation we will have to grow as much food in the first two decades of the new century as that produced over the past 10000 years. Techniques for selecting and upgrading plant products have been refined over centuries of human history to reach current standards of plant breeding (Kung, 1993). However in the present century with the discovery of Mendel's Laws plant breeding rapidly co-evolved with the developing science of genetics. Genetics has provided the breeder with a better knowledge of the processes involved in generating variability, and the means by which such variation may be manipulated in a breeding programme. Genetic advances thus allowed breeders to improve upon the empirical techniques that they had used traditionally. Plant breeding is essentially a selection made by man of the best plants within a variable population. As a result of this selection yields have been increased by improvements in productivity.

Traditionally productivity of a plant has been increased by modifying its morphological characteristics such as the number of kernels per ear in cereals or the weight of individual seeds within the pod of a pulse, and also by modifying physiological traits such as harvest index, the utilization of nutrients, or tolerance to stress. With changing demands on agriculture, objectives are now being altered to

take account of these and new requirements. Quality and nutritive value are now receiving increasing importance, particularly in association with improved efficiency of production (Sánchez-Monge, 1993).

Plant breeding has reached a crossroads. In the past the genetic contribution to yield gains has often been regarded as of secondary importance compared with the improvements through fertilizers, irrigation and extensive use of agrochemicals. Despite this, in the last 50 years it has been estimated that half the yield gains in most major crops are attributable to the introduction of new cultivars as a result of plant breeding. While considerable genetic improvements have been achieved in crops grown under optimum conditions, at least in absolute terms, response to selection is limited in crops grown in marginal situations. At the same time, the need to preserve the environment for future generations, together with new economic constraints, is leading to a more comprehensive and integrated agriculture for which yield alone will no longer be its sole objective (Hayward, 1993).

In order to raise the productivity of marginal land and to be able to exploit new and better husbandry techniques, modern agriculture requires new varieties. But in order to protect a breeder's cultivar and indeed, their own interest, breeders also have to meet certain legislation requirements.

1. 4. 1. Objective of forage grasses breeding

The objectives in grass breeding differ with cereal breeding since with forage grass crops the whole plant represents the yield as low cost feed for livestock. Therefore the ultimate value of a sward should be measured in terms of animal products. Since this is a difficult and costly approach, animal requirements are translated into more specific plant characteristics which are then used as selection criteria.

One basic requirement common to all crops is that the variety should be composed of sufficiently uniform genotypes to ensure varietal stability over

successive generations of seed multiplication and to ensure that it can be recognized with the means usually employed to distinguish individual varieties. In addition, the breeding of herbage crops is concerned, directly or indirectly, with the following:

- Fast growth, after easy and rapid establishment,
- Annual dry herbage matter production and its seasonal distribution,
- Production under high stocking densities,
- Potential for growth in the autumn and spring,
- Long life and compatibility with other species in mixtures,
- Nutritive value (chemical composition, digestibility, and palatability),
- No toxins,
- High soluble carbohydrates,
- Seed production (flowering time),
- Resistance to adverse weather conditions ,
- Persistency,
- Resistance to pest and diseases,
- Tolerance to drought,
- Tolerance to low and high temperatures,
- Winter hardiness,
- Tolerance to unfavourable soil conditions (low nutrients, acidity and salinity),
- Increased responses to high nitrogen levels,
- Easily harvested by animal or machine,
- Harmless to environment ,

(Sneep and Hendriksen, 1979; Sneep, Murty and Utz, 1979; Hodgson, 1981; Oleson, 1987; Eagles and Fuller, 1982; Breese 1983; Laidlaw and Reed, 1993 and Groot *et al*, 1994).

A situation in which all characters need to be improved simultaneously from an initial low level will rarely be encountered. More often, work will be concentrated on species and varieties which are already popular and improvement

will be sought in a few characters which are considered to be limiting. Characters rarely vary independently and care must be taken that improvement in one character is not nullified by a correlated negative response in another trait of agricultural importance (Snoop, Murty and Utz, 1979). Interspecific and intergeneric hybrids may combine favourable attributes from different species, and much effort is spent on crosses much as between different *Lolium* species as well as crosses between species of *Lolium* and *Festuca* (e.g. Lewis, 1982; Thomas and Humphreys, 1991; Suginobu *et al*., 1993; Humphreys and Thomas, 1993; Humphreys, 1994; Humphreys and Ghesquiere, 1994).

1. 4. 2. Domestication, introduction of germplasm and selection procedures

A prerequisite for any effect of selection is of course genetic variability for the favourable attributes. The first step in a breeding programme is therefore to identify germplasm which is expected to fulfil the established breeding goals. Genetic material for breeding could be taken from 1) improved varieties, 2) hybrid progenies, 3) recurrent selection populations, 4) local ecotypes, and 5) gene centres.

The extent of genetic variability within a population, whether a natural population, a breeding population or a variety, depends on the germplasm included as well as the past selection history. Natural populations will usually contain much genetic variability, whereas the variation contained in breeding populations and in varieties is determined by their composition and the intensity of past selection. Synthetic varieties based on few individuals selected intensively will usually be more restricted genetically than, for example open pollinated cultivars (Penny *et al*, 1963 and Hallauer, 1981). When genetic variability is insufficient for specific characters, it may be increased by the introduction of new germplasm from other species or from collections at gene centres. The choice of germplasm for use in breeding will be influenced by the breeding procedure and the time required to achieve the goals. In long term breeding, a very broad basis is desirable in order to

meet future demands. Using a recurrent selection programme and mild selection pressure, unadapted or introduced germplasm may be included in the breeding population.

Van Wijk *et al*., (1993) claimed that forage breeders are often reproached that the genetic improvements in grasses and legumes have not been as great as in many arable crops. This observation can easily be made because the progress in grasses and legumes is more difficult to measure than in arable crops. Far less resources have been offered to grass breeding compared to cereal breeding, while grass crops are generally outbreeders and not inbreeders like most major cereals.

It is clear that a wealth of genetic variation for many characters concerned with productivity is contained within existing varieties. Thus within species variation can be augmented by the collection and introduction of plants from natural populations (Davies *et al*., 1973). Much of this variation is additive and thus can be exploited through breeding programmes (Breese and Hayward, 1972). However a major problem is the establishment of suitable criteria and techniques for selection of the parent plants of improved varieties (Lazenby and Rogers, 1963).

Among the most important examples of artificial introduction of grass species are crested wheat-grass (*Agropyron cristatum*) and desert wheat-grass (*Agropyron desertrum*), introduced into western Canada, and western United States, and both sown across millions of acres of abandoned wheat-fields and over-grazed and drought stricken pasture-land (Hafenrichter *et al.*, 1949). Hafenrichter *et al.* listed several other grass and legumes species (Table 1. 4) which have been tested for their contribution to soil conservation in the Northwest Pacific of the United States, where the summers are hot and dry and the winters mostly mild and humid. Inland and at high elevations, the winters are cold and there is considerable snow. This work could be used as a model for other areas of the world with similar problems.

Table 1. 3. Classification of grasses and legumes of primary importance for soil conservation in the Pacific north-west of the United States (Hafenrichter *et al*., 1949).

	Class	Common name	Scientific name
1	Rapidly developing short-lived grasses	Mountain brome Slender wheat-grass Bearded wheat-grass Tall oat-grass Italian ryegrass Perennial ryegrass	*Bromus marginatus* Nees *Agropyron trachycaulum* (Link) Malte *A. subsecundum* (Link) Hitchc. *Arrhenatherum elatius* J. & C. Presl *Lolium multiflorum* Lam. *L. perenne*
2	Rapidly developing long-lived grasses	Orchard grass Tall fescue Meadow fescue Smooth brome	*Dactylis glomerata* *Festuca arundinacea* *F. elatior* *Bromus inermis* Leyss.
3	Late maturing grasses	Intermediate wheat-grass Pubescent wheat-grass Tall wheat-grass	*Agropyron intermedium* (Host.) Beauv. *A. trichophorum* (Link) Richt. *A. elongatum* (Host.) Beauv.
4	Drought-tolerant long-lived bunchgrasses	Desert wheat-grass Siberian wheat-grass Whitmer beardless wheat-grass Blue bunch wheat-grass	*A. desertorum* (Fisch.) Schult. *A. sibiricum* (Willd.) Beauv. *A. inerme* (Scribn. & Smith) Rydb. *A. spicatum* (Pursh) Scribn. & Smith
5	Drought-tolerant long-lived sodgrasses	Western wheat-grass Thickspike wheat-grass Streambank wheat-grass	*A. smithii* Rydb. *A. dasystachyum* (Hook.) Scribn. *A. riparium* Scribn. & Smith
6	Vernal, dominant dry-land grasses	Big bluegrass (a robust type) Nevada bluegrass	*Poa ampla* Merr. *P. nevadensis* Vasey ex Scribn.
7	Under-storey grasses with heavy root production	Idaho fescue Sheep's fescue Clatsop red fescue	*Festuca idahoensis* Elmer. *F. ovina* *F. rubra*

8	Wet-meadowland grasses	Meadow foxtail Creeping foxtail Reed canary-grass Beardless wildrye	*Alopecurus pratensis* *A. arundinaceus* Poir. *Phalaris arundinacea* *Elymus tritico ides* Buckl.
9	Sand-binding grasses	European beachgrass American beachgrass Volga variety of Siberian (giant) wild-rye	*Ammophila arenaria* Link *A. breviligulata* Fernald *Elymus giganteus* Vahl
10	Native and introduced legumes	Birdsfoot trefoil Big trefoil Cicer milk-vetch Sickle milk-vetch Subterranean clover Flat or everlasting pea Purple beach-pea Perennial vetches	*Lotus corniculatus* *L. uliginosus* Schk. *Astragalus cicer* *A. falcatus* Desf. *Trifolum subterraneum* *Lathyrus sylvestris* *L. japonicus* Willd *Vicia* spp.
11	Well- known legumes commonly used with grasses under sub-humid and humid conditions	Alfalfa White clover Alsike clover Strawberry clover Red clover Sweet clover Common vetch Hairy vetch Austrian winter pea	*Medicago sativa* *Trifolium repens* *T. hybridum* *T. fragiferum* *T. pratense* *Melilotus alba* Desr. *Vicia sativa* *V. villosa* Roth. *Pisum sativum var.* arvense

The success of a breeding programme in meeting its various objectives is dependent upon two main factors. First having available the necessary variation and second, being able to manipulate it to produce a stable new cultivar (Sánchez-Monge, 1993). Gene banks now play an excellent role in maintaining the necessary variation for developing novel germplasm. Breeding via selection was started by early man when he domesticated agricultural plants and has now became a large sophisticated factor with many new techniques.

1. 4. 3. *Lolium* and *Festuca*

Cultivated grasses are less dependent for their growth and survival on close adaptation to the natural environment, since this environment may be substantially changed during the cultivation processes. Hence grasses may be found in cultivation well outside the climatic limits of their natural distribution (Hartley, 1964).

Only a few of the total grass species which are known to occur in nature are used on any important scale as cultivated pasture plants. Hartley and Williams, (1956) estimated the number of important cultivated pasture grass species is about 40 and Whyte, Moir and Cooper (1959) listed 36 grass species. Semple (1970) cited from M.A. Hein (1960), 44 cultivated grasses and their native regions. Many of the grass species have been selected and improved for use in temperate zones, where indeed they are native as is indicated in Table 1. 4. They are also well adapted for making hay.

The *Festuceae* tribe contain one-third of the pasture grasses species listed in Table 1. 4. The two genera *Festuca* (fescue) and *Lolium* (ryegrass) in the *Festuceae* tribe include most of the perennial grasses sown for forage in Europe (Thomas *et al*., 1995). *Lolium multiflorum* and *Festuca arundinacea* are two of four major agricultural grass species within the *Lolium-Festuca* complex of species together adapted to a wide range of conditions and exhibiting complementary characters (Breese *et al*., 1981; Breese and Lewis, 1984, and Thomas and Humphreys, 1991).

Table 1. 4. The principal cultivated pasture grasses belonging to the *Festuceae* (after Hein 1960)

	Common and scientific name	Tribe	Native regions
1	Canada bluegrass *Poa compressa*	*Festuceae*	Europe & Southwestern Asia
2	Cocksfoot or orchard grass *Dactylis glomerata*	*Festuceae*	Europe, North Africa, & temperate Asia
3	Italian ryegrass *Lolium multiflorum* Lam.	*Festuceae*	Southern & Western Europe, Northern Africa, & SW. Asia
4	Kentucky bluegrass *Poa pratensis*	*Festuceae*	Europe, temperate Asia, the Arctic, & North Africa
5	Meadow fescue *Festuca elatior*	*Festuceae*	Europe & South-Western Asia
6	Mountain brome-grass *Bromus marginatus* Nees	*Festuceae*	North-Western U.S.A. & British Columbia
7	Perennial ryegrass *Lolium perenne*	*Festuceae*	Temperate Europe & Asia
8	Red fescue *Festuca rubra*	*Festuceae*	Europe, the Arctic, and temperate Asia
9	Rescue-grass *Bromus catharticus* Vahl	*Festuceae*	South America
10	Rough meadow-grass *Poa trivialis*	*Festuceae*	Europe, temperate Asia, & North Africa
11	Sheep's fescue *Festuca ovina*	*Festuceae*	Temperate and cool-temperate Northern Hemisphere
12	Smooth brome-grass *Bromus inermis* Leyss.	*Festuceae*	Northern & central Europe to China
13	Tall fescue *Festuca arundinacea*	*Festuceae*	Europe, temperate Asia, & Northern Africa
14	Wimmera rye-grass *Lolium rigidum* Gaud.	*Festuceae*	Mediterranean region

1. 4. 3. 1. *Lolium multiflorum* (Italian ryegrass)

The agriculturally important *Lolium* species are *L. perenne*, *L. multiflorum* and to a lesser extent *L. rigidum*. In the United Kingdom *L. perenne* (perennial ryegrass) is a natural climax species of most fertile grazed pastures (Stapledon and

Davies, 1940) and is now generally regarded as the basis of grassland improvement (Breese, 1983). *L. multiflorum* (Italian ryegrass) is used mainly as conservation grass in short term leys. Perennial and Italian ryegrasses are the two most important constituents of highly productive swards (Thomas and Humphreys, 1991) and together account for over 80% of agricultural grass seed sales in the United Kingdom. They are important constituents also of agricultural grasslands in other European countries and have been successfully introduced into temperate areas elsewhere, of which New Zealand is a prime example (Breese, 1983). *L. multiflorum* is a valuable fodder plant for hay or grazing and frequently is an escape from cultivation, naturalized on road sides, field margins, and waste ground (Hubbard, 1980).

The *Lolium* genus probably has its centre of origin in the Mediterranean region (Terrell, 1968). Hubbard (1980) also reported that *L. multiflorum* is a native of central and South Europe, North-West Africa and South-West Asia. However *Lolium spp*. are now widely distributed through temperate areas of the world (Hubbard, 1980 and Breese, 1983).

L. multiflorum, in the past often named *L. italicum* (Hubbard, 1980), is a diploid 2n = 2x = 14 (Thomas and Humphreys, 1991). As pointed out by Breese and Tyler (1986), natural tetraploids have never been reliably reported in *Lolium*, despite its widespread geographic distribution. This is a rather curious fact considering the situation in other genera. However a number of artificial auto-tetraploid and allo-tetraploid types have been produced for agronomic purposes.

L. multiflorum is adapted to long-term survival through seeding rather than vegetative growth, and thus is considered an annual, biennial or weak perennial (Breese, 1983). It is very erect and although capable of tillering, it rarely tillers enough to prevent a steady decline in sward density from establishment onwards. *L. multiflorum* is less winter hardy than *L. perenne*. However, *L. multiflorum* will grow for a long season, starting growth in spring before *L. perenne* and continuing well into the autumn under conditions of high fertility. However, the bulk of growth

from *L. multiflorum* is in the April - June period, making them particularly useful for early spring grazing and a heavy conservation cut. During the summer they tend to run into a seed-head every 35-40 days and this can lower their feeding value at this time. It is particularly useful as a rotation grass, where its two to three years duration is not a disadvantage (Brockman, 1983). Synthetic tetraploid varieties have made a significant contribution to grassland farming, and further efforts are being directed towards breeding cultivars with even better regrowth after cutting, greater persistency and higher quality to give even more flexibility with regard to cutting and grazing management (Thomas and Humphreys, 1991). They are important, as tetraploidy emphasises the natural advantages of *L. multiflorum* in terms of erectness, leaf size and soluble carbohydrate content (Brockman, 1983).

1. 4. 3. 2. *Festuca arundinacea* (tall fescue)

Festuca arundinacea (tall fescue) is a wind pollinated, highly self-infertile polyploid perennial cool-season forage and turf grass. It is used in pastures, parks, lawns, golf courses, football fields, highway medians and road sides. Tall fescue is important for grazing, stabilizing soil for agriculture, and enhancing the environment through multiple uses, such as forage, conservation, and turf. It provides ground cover as a perennial for millions of hectares of erodable land (Buckner and Bush, 1979 and Bacon and Siegel, 1988).

F. arundinacea is a hexaploid (2n = 6x = 42), sometimes named *F. elatior* (Hubbard, 1980). Humphreys *et al.* (1995) by using genomic *in situ* hybridization (GISH) demonstrated that the tetraploid species *F. arundinacea* var. *glaucescens* (FgFg1) contributed two genomes and the diploid species *F. pratensis* (Fp) one, to create the allohexaploid species *F. arundinacea (FpFpFgFgFg1Fg1)*.

Tall fescue is indigenous to Europe (Barnes, 1990), North-West Africa, temperate Asia, and extended into China. Introductions have also been made into

Australia, Canada, Japan, New Zealand, North and South America, and South and East Africa (Hubbard, 1980; Barnes 1990 and Easton *et al*., 1994).

Tall fescue has attained widespread usage as a forage grass for livestock, despite its propensity to cause physiological disorders in grazing livestock (Barnes, 1990). It is more rough and less palatable than ryegrasses and even so then compared with meadow fescue (*F. pratensis*). Its advantage is that it is resistant to cold and drought stress with good persistency, (Thomas and Humphreys, 1991), and once established, growth is aggressive, and plants persist under a wide range of environmental conditions (Burns and Chamblee, 1979).

Tall fescue plants survive under acid (pH 4.7) and alkaline (pH 9.5) soil and are relatively salt tolerant. Growth occurs on soils ranging from low-lying sites with moist, heavy soils to poorly drained and droughted sites (Barnes, 1990). The different native strains occupy distinct habitats, the taller robust ecotypes growing on heavy soils, low-lying meadows and by the sides of rivers and streams, whilst the shorter types are found in grazed pastures, and on drier calcareous and sandy soils in rough hill and down grassland (Hubbard, 1980). Tall fescue is a reliable perennial cool-season grass on some shallow droughty soils. The coarse, deep root system forms a dense sod which also withstands water logging and flooding. Thus, tall fescue stands survive for years, if properly managed. The dense, green turf is resistant to animal traffic and can withstand considerable grazing. Its excellent turf characteristics make tall fescue particularly valuable for industrial parks, lawns, athletic fields, revegetation of roadsides and disturbed areas, and other recreational or conservation purposes (Barnes, 1990).

1.4.4. Polyploidy and potential of species within the *Lolium/Festuca* complex

Between the two *Lolium* and *Festuca* genera, the combination of *Festuca arundinacea* and *Lolium multiflorum* has been claimed to offer the greatest complementation of characters (Breese *et al*., 1981).

The current study will describe the use of anther culture on a *L. multiflorum* × *F. arundinacea* hybrid to combine complementary characters of the parental species. Some of the more important agronomic characters of *L. multiflorum* and *F. arundinacea* are summarised in Table 1.5, taken and modified from Breese and Lewis, (1984) and Thomas and Humphreys, (1991). These characters if combined, could offer good seasonal productivity and nutritional quality with persistence at low input management and tolerance to climatic stress. Italian ryegrass generally provides forage of high digestibility primarily due to higher water soluble carbohydrate than the tall fescue. The tall fescue has better developed root systems and water regulation (Thomas, 1994), which aids persistency and adaptation to extremes of winter cold and summer drought (Thomas and Humphreys, 1991).

The *Lolium* and *Festuca* species may be readily hybridized but all *Lolium spp.* × *Festuca spp.* hybrids are sterile, although there is some evidence of female fertility, since it is possible to obtain backcross progeny when the hybrid is crossed with either parental species (e.g. Reusch, 1959). In an effort to overcome the sterility of the hybrid between these two genera, the chromosome number of intergeneric hybrids has been doubled to produce fertile alloplyploids (Essad, 1956; Buckner *et. al.*, 1961; Lewis, 1966 and Kasperbauer, 1990c). Chromosome doubling can be induced by applying the chemical colchicine, which disrupts spindle formation during cell division so that daughter chromosome sets remain in the same cell and chromosome number is doubled. Generally chromosome doubling results in increase in plant size. From a morphological point of view, polyploids tend to be larger than diploids, with thicker leaves. Another procedure to produce fertile hybrids is the use of auto-polyploid forms of the diploid parents which when hybridized should normally produce fertile allotetraploid hybrids. The cultivars Elmet and Prior were produced at the Institute of Grassland and Environmental Research by hybridizing autotetraploid *Lolium* spp. with autotetraploid *Festuca pratensis* (Lewis, 1982). A population of fertile pentaploid hybrids (2n = 5x = 35) was produced by crossing an autotetraploid *L. multiflorum* cultivar Tetrone (2n = 4x

= 28) with *F. arundinacea* (2n = 6x = 42) from a natural population (Humphreys, 1989).

Table 1. 5. Complementary features of *L. multiflorum* and *F arundinacea* species. More + indicates superior performance.

	Lolium multiflorum 2n = 2x = 14	*Festuca arundinacea* 2n = 6x = 42
Rapid establishment	+++	+
Early spring growth	+++	+++
Summer growth	++	++
Quality	+++	+
Winter hardiness	+	+++
Drought tolerance	+	+++
Persistency	+	+++
Grazing	++	+
Conservation	+++	+++

Polyploidy has featured strongly in plant evolution as a means of conserving favoured hybrid combinations during sexual reproduction. Combining different genomes is likely to extend adaptation rather than increase yield *per se* (Breese *et al*., 1981). Interspecific and intergeneric hybridization of plants has been intensively used for plant breeding and also for evolutionary studies (Kleijer, 1984). The following two methods are the most important approach for conventional plant breeding.

1) Introgression - the transfer of one or a few specific genes from one species to another. Many successful examples are known (Lacadena, 1978).

2) The creation of allopolyploids in an attempt at combining the good characteristics of both parents. These may be entirely new forms, or the

recombination of existing allopolyploids for the introduction of new genetic variation (Kleijer, 1984).

Many agronomicaly and economically important crop plants such as wheat (*Triticum astivum*), oats (*Avena sativa*), cotton (*Gossypium* spp.), tobacco (*Nicotiana tabacum*) and tall fescue (*F. arundinacea*) are allopolyploid. There are few examples of success in breeding newly made allopolyploids for use in agriculture. One of the best examples is the combination of *L. multiflorum* and *L. perenne* (Breese and Lewis, 1984). Kleijer, (1984) cited a few examples which have been tested on large scale, which were inferior to the parental species or the corresponding natural forms. They were: resynthesized *Brassica napus*, *Raphanobrassica* (the amphidiploid of *Raphanus sativus* and *Brassica oleracea*, (McNaughton and Ross, 1978) and the amphidiploid of *L. perenne* or *L. multiflorum* Lam. and *F. pratensis* Huds. (Griffiths *et al*., 1979). The most successful synthetic allopolyploids cultivated on a large scale are triticale (wheat-rye amphiploid) (Gupta and Reddy, 1991) and as cited earlier tetraploid hybrid ryegrass (*L. multiflorum ´ L. perenne*) (Breese and Lewis, 1984).

Attempts to produce *Lolium* × *Festuca* amphiploid hybrids for use in agriculture have thus far failed (Humphreys pers. comm.). Early indications that chromosome pairing in amphiploids of *L. multiflorum ´ F. arundinacea* and in the amphidiploid *L. perenne* or *L. multiflorum ´ F. pratensis* was restricted to homologues of each species. Hybrids were selected with high levels of preferential chromosome pairing, a prerequisite for improved stability (Lewis, 1982 and Breese and Lewis, 1984). However a satisfactory level of stability in the amphiploids has not been achieved and the occurrence of multivalents and univalents has resulted in chromosome loss, chromosome recombination and subsequent genetic instability (Lewis, 1966; Lewis, 1982; Kleijer and Morel, 1984). Agronomically, the predominant weakness of the amphiploid between *L. multiflorum* and *F. arundinacea* was slow germination and poor initial seedling growth, although the amphiploid was superior in seedling growth to tall fescue (Diikstra and Devos,

1975). However, improved palatability of the *L. multiflorum* × *F. arundinacea* amphiploids compared with tall fescue was reported by Webster and Buckner (1971). Before such hybrids can be used commercially, improvements in genetic stability will be necessary.

1. 4. 5. Introgression lines

An alternative to producing genetically stable amphiploids is to introduce a limited number of genes from one species into another by recombination between the parental chromosome sets (Humphreys, 1989). Gene transfer has been accomplished through backcrossing fertile hybrids of *Hordeum vulgare* and *H. spontaneum*. Another example is tomato which has been hybridised with related wild species to introduce many genes conferring disease resistance (Thomas, 1993).

There are indications that in breeding for improved tolerance to environmental stress, there may be advantages of introgressing a limited number of genes from one species into another rather than combining complete genomes which may produce genetic imbalance and thus decrease the adaptive response (Thomas and Evans, 1990 and Humphreys *et al*., 1993).

If a character is semi-quantitative and if the favourable alleles are not dominant, traditional backcrossing methods may not be quite so efficient. In such cases marker assisted selection (MAS) is necessary to aid selection of the required alleles (Stuber, 1989). The utility of marker assisted selection is based upon the establishment of a linkage relationship between an easily identifiable major gene marker and a character of agronomic importance. Marker assisted selection is based upon the principle that if a gene (or block of genes) is linked to an easily identifiable genetic marker it may be more efficient to select in a breeding programme for the marker than for the trait itself (Hayward *et al*., 1994). Brown *et al*. (1989) used isozymes for MAS in a backcross breeding programme involving *Hordeum* to increase barley yield.

Clearly in amphiploids homoeologous chromosome pairing is a severe disadvantage but it is an essential prerequisite for any introgression programme aimed at introducing genes from one species into another. An understanding of the meiotic chromosome behaviour and an ability to regulate the mechanisms which control chromosome pairing in the *Lolium/Festuca* complex will offer plant breeders a choice of which breeding procedure to use.

As in wheat and oats a gene or genes exist in hexaploid *F. arundinacea* which determine the strict homologous chromosome pairing characteristic of this amphiploid species (Jauhar, 1975). Jauhar proposed that this gene or genes are ineffective when hemizygous but in the homozygous condition, regulate chromosome pairing and confine it to homologous associations. In hybrids with *Lolium* species, genetic regulation due to the gene(s) being ineffective in the hemizygous condition, breaks down (e.g. Kleijer, 1984 and Evans and Aung, 1986) and the opportunity for interspecific chromosome pairing and recombination arises.

Genes have also been identified in *Lolium perenne* which reduce the extent of homoeologous chromosome pairing in hybrids between these *L. perenne* (*Lp19*) genotypes and *F. arundinacea* (Evans and Aung, 1986). There was evidence that in the F_1 hybrid, the *L. perenne* pairing genes complemented the system in tall fescue to reduce association between non-homologous chromosomes.

To enable the successful transfer of genes from one species to another, as high a level of interspecific chromosome paring as possible is required. A pentaploid hybrid was formed by the hybridization of tetraploid *L. multiflorum* (2n = 4x = 28) and *F. arundinacea* (2n = 6x = 42) (Humphreys, 1989). A high level of intergeneric chromosome association has been demonstrated in such pentaploids and moreover they have been found to be fertile (Thomas *et al*., 1983; Kleijer, 1984 and Kleijer and Morel, 1984). The frequency of homoeologous chromosome pairing in *L. multiflorum* × *F. arundinacea* hybrids (Kleijer, 1984; Thomas *et al*., 1983; Carnide *et al*., 1993) would indicate that a backcrossing programme with one of the parental species as recurrent parent may provide a means through gene introgression

for improved quality and/or disease resistance and adaptation to environmental stress of the parental genotype.

Although a 42 chromosome cultivar, "Kenhy", with certain Italian ryegrass characters created by backcrossing a tall fescue × Italian ryegrass hybrid to the fescue (Buckner, Burrus *et al.*, 1977) has been marketed, the shortage of suitable genetic markers has until recently restricted any controlled evaluation of the alternative introgression breeding approach.

In a backcrossing programme involving *L. multiflorum* (the recurrent parent) and *F. arundinacea*, the diploid *L. multiflorum* phenotype was rapidly recovered (Humphreys, 1989) with the inclusion, in some progeny, of a small number of genes from the fescue parent. In field drought trials derivatives of these backcross populations were on average less drought resistant than the *L. multiflorum* parental population, but 3% of individuals were as drought resistant as *F. arundinacea*. After only one cycle of selection and polycrossing of these drought resistant *Lolium*-like plants, the mean drought resistance of most progeny lines was significantly improved, in some cases to near that of *F. arundinacea* (Humphreys and Thomas, 1993 and Humphreys, 1995). Using genomic *in-situ* hybridisation (GISH), an introgressed *F. arundinacea* chromosome segment on a *Lolium* chromosome was identified and strongly indicates that this improved drought resistance was due to the transfer of genetic material from *Festuca* into *Lolium* (Thomas *et al.*, 1995)

The selected trait may be controlled by a single major gene such as in the case of disease resistance, or, under the control of many genes each of small effect. In forage grasses we have very few important traits controlled by a single locus (Hayward *et al.*, 1994). Traits such as drought resistance are polygenically controlled, and genes which determine such a character may well be located on chromosomes in each of the three homoelogous sub-sets of chromosomes that make up the genome of *F. arundinacea* (Humphreys and Ghesquiére, 1994). Introgression lines are particularly useful where the introduced trait is controlled by very few

genes or by a linked block of genes, where a segment of the genome behaves in the same manner as a single major gene.

1. 4. 6. Application of biotechnology in plant breeding

Projected population increases, possible climatic changes through greenhouse effect and alteration in dietary habits for health or fashion reasons have all put the plant breeders under pressure to produce more food, different types of food, or food with a more acceptable flavour. Plant breeding has always been a long term activity but now it is expected to achieve these new goals even more quickly than before (Collin, 1995). Plant improvement by breeding is severely restricted by the availability of a rather limited gene pool owing to natural incompatibilities, even between related species, and by the time scale of most breeding programmes (Vasil, 1995).

Therefore, much attention has been directed recently to the newly emerging and novel technologies of plant cell and molecular biology (biotechnology), which provide a powerful means to supplement and complement the traditional methods of plant improvement, by permitting access to an unlimited gene pool through the transfer of desirable genes between any two species of interest, irrespective of evolutionary or taxonomic relationships (Vasil, 1995). It became apparent that traditional and new technologies should complement each other in order to be most useful in developing superior new genetic lines, hybrids, or cultivars. It was also apparent that the first step in such an approach should be to identify real problems that are difficult or extremely slow to solve by traditional methods. The next step should then be to consider the various new technologies in term of their potential to solve the problems (Kasperbauer, 1990a).

It has been maintained that through these techniques new variation can be created which will provide resistance to drought, low temperature, pests and diseases, yields will be increased and flavours changed. All this will be achieved by

the regeneration of tissue cultures to produce somaclones, protoclones, anther cultures and by the practice of protoplast fusion, cybridisation, gene transfer, plant transformation and micropropagation. Most of these techniques are available for the major food crops, such as the cereals and the legumes and the techniques are used routinely on these crops in many laboratories of the world. However there are limitations to the general application of these techniques to plant breeding. The most serious problem is that there are only a few genotypes which have been regenerated from tissue culture and transformed by genetic engineering and many of these are selected for their ease of manipulation rather than their commercial value. The other major problem is that all of the techniques and particularly those of molecular biology are very expensive to apply to crop improvement. The reality is that the practising plant breeder has a limited budget and is often working with local or national varieties.

1. 4. 7. Haploid production and its significance

The significance of haploids for plant improvement, and as a tool in various disciplines of plant science has been widely reviewed (e.g. Kasha, 1974; Poehlman, 1987; Alejar *et al*., 1995 and Wenzel *et al*., 1995). Since haploids are of great importance, especially in studies of the induction of mutations and for the production of homozygous lines, they are widely produced. However, the conventional methods (Kimber and Riley, 1963 and Magoon and Khanna, 1963), employed by plant breeders for their production are cumbersome, laborious and not very efficient. With the introduction of techniques for the induction of androgenesis by the culture of excised anthers (Guha and Maheshwari, 1964), isolated pollen (Nitsch, 1974) and by chromosome elimination through hybrid embryo culture (Kasha and Kao, 1970 and Kisana *et al*., 1993) it has become increasingly evident that tissue culture methods could speed up the production of haploids for breeding programmes.

1. 4. 7. 1. Application of haploids

Haploids may be used in plant breeding and in genetic studies as follows:

1. The rapid production of homozygous lines, and more reliable and effective selection (Morrison and Evans, 1988 and Foroghi-Wehi and Wenzel, 1989).

2. The development of doubled-haploid lines from hybrid material. .The rapid homozygosity can mean cultivar release can be brought forward by up to four years thereby saving research costs and providing increased returns to growers (Chu, 1982; Baenziger *et al*., 1984; Snape and Simpson, 1984; Brennan, 1989 and Luckett and Smithard, 1992). Many new varieties have been produced using androgenesis e.g. in barley (Foroghi-Wehi and Friedt, 1984); wheat (De Buyser *et al*., 1987) and rice (Huang *et al*., 1988 and Alejar, 1995).

3. Reduction at the polyploid level: In tetraploids, like *Medicago* and potato, haploidization is used to produce diploids. In several tetraploid crops it has become possible, by this approach, to combine wild species with cultivated forms at the diploid level (Foroghi-Wehi and Wenzel, 1993).

4. Production of variations in recombinant lines following interspecific and intergeneric hybridization (Kasperbauer, 1990c).

5. Use of haploids in mutation studies. A recessive mutation will be observed immediately in a haploid plant as the genotype can be identified from the phenotype. In a homozygous diploid plant, a recessive mutation of a dominant gene will be covered by the corresponding dominant allele and the mutation will not be observed until segregation has brought two recessive alleles together in a later generation (Poehlman, 1987).

6. Easier selection criteria in a small populations in haploids than at diploids and polyploids, because of simpler genetic structure (Kasperbauer, 1990b).

7. Obtaining variation in chromosome number such as monosomic series for cytogenetic studies.

8. Different combinations of quantitatively inherited characters. Although there is benefit from androgenesis in rapid incorporation of a specific trait, much more important are strategies which allow the combination of quantitatively inherited characters (Wenzel *et al*., 1995).

9. Haploids of a polyploid species (polyhaploids) may be used to transfer genes from the poyploid to related diploid species (Poehlman, 1987).

10. In species with incompatibility alleles, in which self-pollination is restricted, doubled haploids offer a possibility for producing plants with homozygous alleles (Poehlman, 1987).

11. Use of doubled-haploid lines in genome mapping. In a population of doubled-haploid lines the identification of phenotypic markers is more efficient, as intermediate expressions due to heterozygosity are excluded (Foroghi-Wehi and Wenzel, 1993).

12. Increasing progress in somatic hybridization, e.g. in potato, the production of haploids becomes more essential resulting in a broad haploid population as fusion plants (Wenzel *et al*., 1995).

13. Increasing the efficiency of plant breeding: The use of double haploid lines greatly reduces the genetic complexity of progeny, since only homozygous genotypes occur (Wenzel *et al*., 1995). The size of populations of progeny which is screened for a desired genotype can be reduce accordingly. For example, in order to select a specific recombination of five genes with a probability of 95%, about 3000 F_2 plants have to be screened compared to 100 F_1 derived doubled haploid lines (Graner and Wenzel, 1992).

14. Molecular genome identification: Haploids due to their simpler segregation patterns are an important prerequisite to identify genes (Wenzel *et al*., 1995; Westecott and Huang, 1995). Most Restriction Fragment Length Polymorphism (RFLP) maps of barley are based on such double haploid

populations. Particularly for Quantitative Trait Loci (QTL) analysis double haploid populations are an important tool to produce reproducible DNA-polymorphisms (Wenzel *et al*., 1995).

15. In transformation

1. 4. 7. 2. Haploid production procedures

Several techniques for producing and evaluation haploids have been proposed with different crops. These include such widely different procedures as:

1.4.7.2.1. Twin seedling

Haploid plants can occur as natural accidents. In some strains of maize, about one kernel out of each 1000 (Poehlman, 1987) will have an embryo with the haploid chromosome number. The occurrence of haploids via twin seedlings varies in frequency among genotypes, even within the same species (Malik and Tripathi, 1970). The natural occurring haploid plants may be recognised easily by utilization of suitable marker genes. Many of the haploids will grow to maturity, and about one out of each ten can be self-fertilized successfully to give a diploid progeny (Poehlman, 1987). Haploid plants are usually lost because they are smaller than normal seed-borne plants and most or nearly all of them are sterile.

1. 4. 7. 2. 2. Semigamy

Haploid plants occur naturally at a very low frequency in cotton. Haploid plants may be identified from diploid plants by smaller plant parts, zigzag stems, and lack of pollen shedding. Haploid plants of selected parentage may be produced by the use of semigamy. Semigamy is an abnormal type of fertilization

in which a male gamete fertilizes an egg, but does not fuse with the egg nucleus. Both sperm and egg nuclei divide independently, so that the embryo contains separate sectors of paternal and maternal tissue. By doubling the chromosomes, fertile homozygous diploid plants may be obtained (Poehlman, 1987).

1. 4. 7. 2. 3. Maternal haploids by chromosome elimination

Haploid plants have been derived by intergeneric hybridization and then elimination of male chromosomes in several crop plants. For example, haploid embryos develop on barley (*Hordeum vulgare*) plants after pollination by certain *H. bulbosum* lines (Kasha and Kao, 1970; Lange, 1971a,b and Kasha and Sadasivaiah, 1971). Haploid plants of wheat were produced by intergeneric hybridization between wheat (*Triticum astivum*) and *H. bulbosum* after chromosome elimination of *H. bulbosum* (Barclay, 1975; Inagaki and Snape, 1982).

Other examples include intergeneric hybridization of wheat and maize. Haploids, of hexaploid wheat (*Triticum astivum*) (Kisana *et al.*, 1993) and of tetraploid wheat genotypes (Sarrafi *et al.*, 1994) were produced after elimination of maize chromosomes. In both cases F_1 wheat hybrids were crossed with maize and all the regenerated plants were haploids (2n = 21 chromosomes).

1. 4. 7. 2. 4. Parthenogenesis

Isolation of unfertilized egg cells to regenerate haploids *in vitro* is known as parthenogenesis. In several crops it is possible to induce parthenogenesis by *in vitro* culture of unpollinated ovaries and ovules (Yangan Zhou, 1982). Haploid embryo sac cells are able to develop into haploid embryos or calli *in vitro* (Foroghi-Wehi and Wenzel, 1993). Parthenogenetic haploid production in *Triticum aestivum* and *Nicotiana tobacum* (Zhu and Wu, 1979), *Gerbera*

jamesonii (Sitbon, 1981), and *Zea mays* (Ao *et al*., 1982) *Citrus sinensis* (Kochba and Spiegel-Roy, 1982), *Beta vulgaris* (Hosemanns and Bossoutrut, 1983) *Helianthus annus* (Cai and Zhou, 1984) *Cucurbita pepo* (de Vaulx and Chambonnet, 1986) *Hevea brasiliensis* (Chen *et al*., 1988) and *Allium cepa* (Muren, 1989) has led to the production of haploid green plants.

Until now the practical application of parthenogenesis has been limited by the low frequency of haploid induction in most species, except for potato (Foroghi-Wehi and Wenzel, 1993). In addition parthenogenesis is normally more laborious than androgenesis since it demands both intensive greenhouse work and *in vitro* equipment (Wenzel *et al*., 1995). Only in plant species where anther culture has been unsuccessful as in sugar beet (Speckmann *et al*., 1986) or onion (Muren, 1989) is haploid parthenogenesis from unpollinated ovules or ovaries of practical interest.

1. 4. 7. 2. 5. Androgenesis

The first successful induction of haploids from young anthers cultured *in vitro* was described by Guha and Maheshwari in 1966. In mature pollen grains the nucleus has already undergone an unequal division, and are normally too old to induce desired somatic development into haploid plants, the unincleate condition of the microspore is normally necessary for successful anther culture. The development of the microspore to a haploid sporophtye, is called microspore androgenesis or simply androgenesis. A androgenesis occurs only under *in vitro* conditions (Foroghi-Wehi and Wenzel, 1993).

Androgenic haploids have been obtained successfully in numerous plant species by using anther culture, microspore culture, or floret culture techniques. Nowadays it has become such a reliable and useful tool in plant breeding that private laboratories are adopting this technique for commercial production.

1.4.7.2.5.1. Anther culture

Anther culture refers to the *in vitro* culturing of anthers containing microspores or immature pollen grains on a nutrient medium for the purpose of generating haploid plants (Poehlman, 1987). Doubled-haploid plants are homozygous at all loci. Lines generated by the doubled-haploid procedure reach preliminary yield trials two to three generations earlier than with classical crossing methods like pedigree selection or bulk-selection. It is even possible to evaluate anther culture derived lines at the haploid or polyhaploid stage (Kasperbauer, 1990b,c) either *in vitro* or *in vivo*.

A report by Guha and Maheshwari (1964) demonstrated that anther culture of *Datura innoxia* can yield haploid plants. Of the many methods that have been proposed for raising haploids in higher plants, anther culture has been the most promising in terms of being both effective and of general application (Clapham, 1977). There has been extensive research on culturing anthers of various species of plants. In the *Gramineae* most progress in anther culture has been made with the self-pollinating cereal species, rice, triticale, wheat and barley. Some efforts are being made with the out-breeding species rye and maize at improving anther culture. For most other monocotyledonous species, response in anther culture is poor and variable, and the majority of plants obtained were albinos (Olesen, 1987). Table 1. 6. shows some results of grass species that have been obtained from anther and pollen culture.

Irrespective of the early events in the division of the microspore nucleus there are two modes of androgenesis, the direct and the indirect, which are diagammatically represented in Figure 1.1.

Direct androgenesis: in this type the microspore behaves like a zygote and undergoes various stages of embryogeny simulating those *in vivo*, as in *Atropa*, *Datura* and *Nicotiana*. The embryos, mostly at the globular stage, are released

from the exine and develop further. Finally the cotyledons and plantlet emerge from the anthers in 4-8 weeks.

Table 1. 6. Grass species from which results have been obtained from anther and pollen culture. The references given are not necessarily the first reported. Al: albino plantlet, Gr: green plantlet, Pl: plantlet (no information about colour), R: only root development, C: macroscopic callus, E: embryos (in some cases only microscopic, a: pollen origin not confirmed. (continued on next page)

Species	Results	References
Aegilops caudata × *A. umbellulata*	Al	Kimata & Sakamoto, 1972
Aegilops umbellulata	E	Zenkteler *et al*., 1975
Agropyron repens	E	Zenkteler *et al*., 1975
Bromus inermis	E	Zenkteler *et al*., 1975
Dactylis glomerata (panicle culture)[a]	Al & Gr	Conger & McDonnell, 1982
Festuca arundinacea (anther culture)	C	Niizeki & Kita, 1973
Festuca arundinacea (panicle culture)	Al	Kasperbauer *et al*., 1980
Festuca pratensis	Al	Rose *et al*., 1987a
Festuca pratensis (2x) × *Lolium multiflorum* (2x)	Al	Neitzche & Wenzel, 1977
Festuca pratensis (4x) × *Lolium multiflorum* (4x)	Gr	Neitzche & Wenzel, 1977
Lolium multiflorum	Al & Gr	Pagniez & Demarly, 1979
Lolium multiflorum	Al	Rose *et al*., 1987a
Lolium multiflorum (2x) × *L. multiflorum* (4x)	Al & Gr	Ward, 1993

Lolium moltiflorum (4x) × *Festuca arundinacea* (12x)	Al & Gr		Neitzsche, 1970
Lolium perenne (2x) × *L. perenne* (4x)	C		Ward, 1993
Lolium perenne × *Festuca pratensis*	Al & Gr		Rose *et al.* 1987a
Lolium perenne	Al & Gr		Stanis & Butenko, 1984 & Opsahl-Ferstad & Rognli, 1994
Phleum pratense	R & C		Niizeki & Kita, 1973
Secale cereale	Al		Wenzel & Thomas, 1974 and Daniel, 1993

Indirect androgenesis: in contrast to the direct androgenesis, the microspores instead of undergoing embryogenesis, divide a few times to form a callus which bursts through the anther wall. This mode of development is quite common and is usually caused by complex media, and in cases where the polarity seems to be disturbed. The callus either differentiates to form embryos, or roots and shoots on the same medium, or it has to be transferred to another regeneration medium for embryogenesis. The callus-derived plants are mostly undesirable as they exhibit genetic variations and polysomy (Reinert and Bajaj, 1977). Green plants can then be transferred into soil. Androgenic plants can be evaluated either at the haploid/polyhaploid stage, and/or after doubling the chromosomes spontaneously or chemically by using colchicine.

1.4.7.2.5.1.1. Anther culture of *L. multiflorum* × *F. arundinacea* pentaploid hybrid

This thesis will describe results from anther culture of an intergeneric hybrid between *Lolium multiflorum* and *Festuca arundinacea*. It was proposed to explore anther culture as a means of revealing and selecting a wide range of gene combinations controlling some of the more important agronomic and complementary traits of the two parent species.

The *L. multiflorum* × *F. arundinacea* (*Lm/Fa*) pentaploid hybrid was used in this project was selected because of:

1. The possibilities for selecting novel gene combinations.

2. Morphology of the parent genotype: a) Fertile anthers and large inflorescence (Humphreys, 1989) permit anther culture of F_1 intergeneric hybrids. b) Some important complementary characters of the two parental species (see Table 1.5).) are expressed in the hybrid i.e. less fibrous, and higher tillering than *F. arundinacea;* excellent persistence, drought and cold tolerance compared with *L. multiflorum*.

3. Previous evidence of high response to tissue culture: Good regeneration from cell suspension and callus culture (Humphreys and Dalton, 1992).

4. Evidence for somaclonal variation from cell suspension culture including somatic recombination (Humphreys and Dalton, 1992).

5. High frequency of interspecific chromosome recombination between the parental species (Humphreys, 1989; Humphreys and Thomas, 1993; Humphreys, 1995 and Thomas *et al*., 1995).

6. A known genetic marker on each of the five chromosome sets in the hybrid i.e. 5 homoeoalleles: The *Lm/Fa* pentaploid hybrid has been characterised for the isozyme phosphoglucoseisomerase (PGI/2) locus. It carries PGI/2

homoalleles **a** and **b** from *L. multiflorum*, and PGI/2 homoeoalleles alleles $\mathbf{a}^{+}$, **c** and **e** from *F. arundinacea* (Humphreys and Ghesquiere, 1994).

It was hoped that through anther culture, novel genotype/s would be produced carrying a range of complementary traits which are particularly important under today's intensive grassland farming systems, such as rapid establishment, early spring growth, forage quality and quantity, winter hardiness, drought tolerance, and persistency. Anther culture will be assessed as a method to more rapidly and effectively select the characters described above in comparison with conventional breeding procedures.

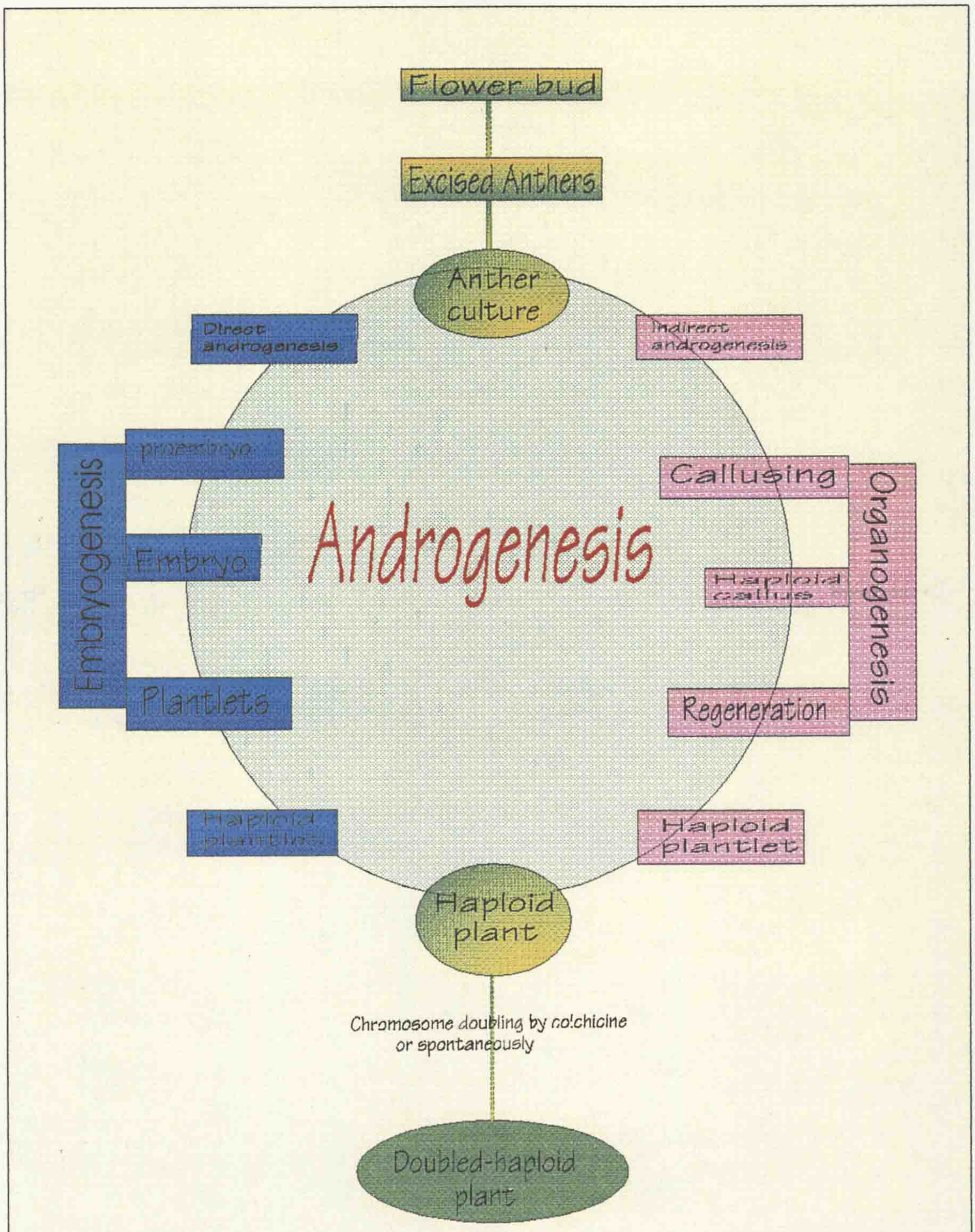


Fig. 1. 1. Schematic representation of the excised anthers and the development of haploid plants directly by embryo formation, or through haploid callus (Taken and modified from Reinert and Bajaj, 1977)

CHAPTER TWO

GENERAL MATERIALS AND METHODS

The aim of this chapter is to present a general account of the materials and methods used in this project. More specific details are described within the relevant chapters.

2.1. Construction of the *Lolium multiflorum* × *Festuca arundinacea* pentaploid hybrid.

The plant material used for anther culture was a pentaploid hybrid *Lm/Fa* (2n = 5x = 35) provided by Dr. M. W. Humphreys from the Institute of Grassland and Environmental Research (IGER), Aberystwyth. The hybrid was derived from hybridizing an autotetraploid *Lolum multiflorum* (*Lm*) (2n = 4x = 28) plant and allohexaploid *Festuca arundinacea* (*Fa*) (2n = 6x = 42), *Accession No.* Bn 949, collected from a natural Swiss population (Figure 2.1).

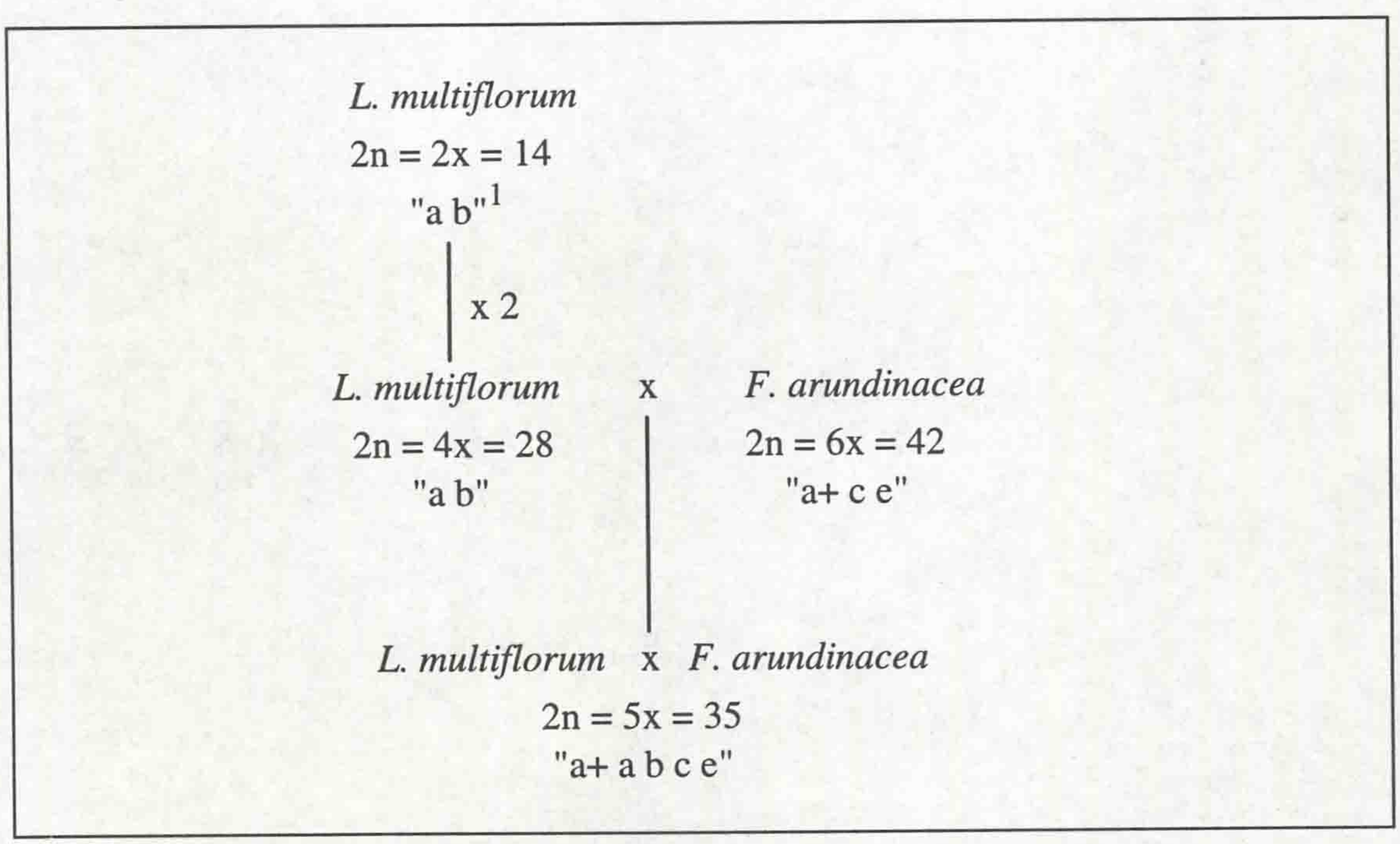


Figure 2.1: Derivation of *Lm/Fa* pentaploid hybrid based on isozyme variation.

[1] Indicates PGI/2 alleles.

The hybrid was constructed using *Lolium* and *Festuca* parents with different phosphoglucoisomerase (PGI/2) alleles. The *Lolium* parent had a phenotype "ab" and the *Festuca* parent had a phenotype "a^{+}ce". The F1 hybrid had a PGI/2 phenotype "a^{+}abce" with each PGI/2 allele labelling a different homoeologous chromosome in the pentaploid hybrid.

The F1 pentaploid hybrid (*Lm/Fa*) was sufficiently male fertile (50% fertility) (Humphreys 1989) to be used as a male parent in a backcrossing programme with *L. multiflorum* (Humphreys and Ghesquière 1994). The anthers of the pentaploid hybrid were well formed and considered suitable for anther culture.

2.2. Anther culture

Anthers of the *L. multiflorum* × *F. arundinacea* (5x) pentaploid hybrid at the uninucleate stage were isolated from flowering shoots and incubated on a modified Murashige and Skoog (MS) medium under lights at 25°C. Following callus initiation, anthers were transferred to a regeneration medium for embryo and shoot formation. Once the regenerants were sufficiently well developed, they were transferred to compost. More than 2,300 plantlets were produced through androgenesis, of which 507 anther culture derived (ACD) plants were established in soil.

2.3. Drought stress experiment in the field at IGER, Aberystwyth.

From established anther culture derived (ACD) plants, 88 plants were selected for assessment of drought resistance based on whether the plant had received:

i. A vernalization treatment for a minimum of 62 days. *F. arundinacea* must be vernalized to flower at temperatures less than +6°C for at least two months.

ii. The genotypes were different and originated from different source (i.e. the panicle, anther, callus, plantlet, etc.). Only ACD plants which had different tissue culture history were selected to remove any possibility that more than one regenerant was derived from the same pollen grain and thus were the same genotype.

iii. The morphology, based on plant vigour, leaf size, and plant shape and growth habit was different. ACD plants were chosen to establish the full extent of variation in morphology to establish whether plant growth habit was related to drought resistance.

Other plants used as controls were; four plants of *L. multiflorum* cultivar 'Tribune', to represent the drought sensitive parental species in the hybrid, four plants of *F. arundinacea* Bn 949, as drought resistant parent of the hybrid and four ramets of the original *L. multiflorum* × *F. arundinacea* (5x) hybrid. The total number of genotypes used in the field drought experiment was therefore 97 (100 plants).

All 100 plants were divided into five equal clonal parts prior to planting in the field and each was established in pots in an unheated glasshouse at IGER, Aberystwyth. Replicate clones of each genotype were transferred to the field in a completely randomized split plot design (main plots = environments, sub plots = genotypes) on 15 May 1994. An experiment was designed with four clonal replicates of each genotype, leaving one clonal part for other studies (for example, see 2.4). Groups of two replicates were planted in the field in two blocks about five meters apart. One barrier row of the parental population as guard plants were grown around each block. The distance between plants was 30 cm between rows and 20 cm within rows. No fertilizer

was supplied to emphasise variation in plant vigour under stress conditions. All plants were irrigated regularly to ensure good establishment.

2.4. Freezing tolerance test (carried out at IGER, Aberystwyth)

Clonal parts of ACD plants, used in the field drought experiment described in 2.3, with sufficient tillers (47 ACD plants) to allow calculation of the lethal freezing temperature where 50% of tillers within a genotype are destroyed (LT_{50}), were selected for testing their freezing tolerance. The same genotypes which previously had been used in the field drought experiment, were selected as controls i.e. four plants of *L. multiflorum* cultivar 'Tribune', as freezing susceptible parent species in the hybrid, two out of the four plants of *F. arundinacea* Bn 949 as a winter hardy parent of the hybrid, and four ramets of the original *L. multiflorum* × *F. arundinacea* (5x) hybrid. The totai number of genotypes used in the freezing tolerance experiment was 54 (57 plants).

All 57 plants were cold acclimated for two weeks in a growth room at 2 °C and 8 hours light. Individual tillers from each genotype were then exposed to freezing temperatures (from -2°C to -14°C) in a polyethylene glycol (PEG) tank. When the PEG temperature dropped to the appropriate temperature, 5 tillers were taken form each genotype and planted in John Innes Potting Compost and left to recover in a growth room at +15°C and 8 hours day length. The LT_{50} of each genotype was calculated based on the percentage of surviving tillers which were green and showing signs of regrowth after three weeks recovery.

2.5. Glasshouse drought experiment (carried out at IGER, Aberystwyth)

A glasshouse experiment was designed to emphasise genotypic difference in drought tolerance between ACD plants by creating more

uniformity in plant size and to minimize interference from environmental factors which may have affected the response to drought stress in the field experiment described in 2.3. From the 88 ACD plants used in the 1994 field experiment, 57 had sufficient tillers to be used for a second drought test in 1995. The *Lolium*, *Festuca* and pentaploid hybrid genotypes used as controls in the field experiment were used again as controls. In addition, 71 other ACD plants were included in the glasshouse drought experiment. The additional plants were selected as different genotypes based on their different origin in culture and contrasting morphology (based on plant vigour, leaf size, plant shape and plant growth habit). Ten tillers were taken from each genotype (140 plants including controls) to be drought stressed. They were split on 20th April 1995 and each tiller grown separately to produce 10 replicate clones which were maintained in multitrays in an un-heated glasshouse prior to planting in soil in deep brick bins in a glasshouse. The 140 plants to be used in the drought experiment were randomized and planted accordingly in one of ten brick bins containing 1 m^2 homogeneous soil in an unheated glasshouse on 7th June 1995. Each bin represented one replicate of the drought stress experiment (10 replicates). One barrier row of a mixture of three diploid Italian ryegrass varieties Titania, Trident and Tribune were grown as guard plants around each replicate block. The distance between plants was 8 cm both between and within rows. No additional fertilizer was supplied. When the genotypes were established (after two weeks) all replications except two (IV and IX which were irrigated daily) were droughted for 77 days. Following the end of the 77 days drought period, the condition of each plant was assessed using a modification (see Chapter 5) of the visual scale of 0 to 5 based on proportion of living green plants (as described by Humphreys and Thomas, 1993). The plants in all 10 bins were subsequently irrigated daily over the following three week period to allow recovery. The recovery condition of each plant was scaled from 0 to 6, again based on proportion of living green plant and signs of

regrowth. In both the drought and recovery, scores of 0 represented death and scores of 5 (or 6) represented the growth condition typical of the most stress tolerant *F. arundinacea* control.

2.6. Cell membrane damage

Cell membrane damage of ACD plants (Blum and Eberion, 1981) was measured by imposing desiccation or temperature stress on detached lamina segments and measuring electrolyte leakage.

When sufficient shoots had been produced, circa 50 mg healthy, young and clean leaf tissue of each genotype was sealed over dry silica gel in 25 ml plastic "sterilin" tubes. After 17 hours, the leaf tissue was placed in boiling tubes with 10 mls of deionised water and 0.05% of organic non-ionic detergent (TRITON) for 4 hours at room temperature to allow leakage of solutes released by damage to the membranes. The detergent was used to lower surface-tension so that the samples would submerge in the water. Because the detergent used in this test was nonionic, it did not change the conductivity of the water.

Solute leakage was estimated by increase in water conductance. The electrolyte conductivity (C1) was measured by a dip-cell and Russell CD 800 Conductivity/Resistivity Meter. The samples were then autoclaved under pressure of 1 kg/cm^2 at 121°C temperature for 15 minutes to rupture all of the membranes and release all the electrolytes. After cooling, the electrolyte conductivity (C2) was measured. The relative cell membrane damage was calculated as: (C1/C2) × 100. Two replicate samples of each genotype were used on each of two dates.

In a preliminary test, samples of a total of 7 ACD plants and the three control genotypes were desiccated for 16, 24, and 48 hours and cell membrane damage calculated as explained above. This preliminary test was designed to find the best desiccation period for 50 - 75% relative cell membrane damage.

Any leaching from un-stressed tissue was considered minimal and thus was ignored. The leaf width and thickness of ACD plants varied, thus calculations were made from leaf samples of equal weight and not simply from leaves of equal length.

2.7. The stomata distribution on the leaf surfaces

Transpiration in plants takes place through the stomata. Thus the density and the distribution of the stomata on the leaf surfaces may influence plant tolerance to environmental stresses such as drought and heat tolerance and has been used as a selection criterion in grasses (Gay, 1994). In order to assess possible influence of stomatal distribution on stress tolerance in ACD plants, the density of stomata and the ratio of stomata on the adaxial and abaxial leaf surface of some ACD plants with contrasting drought tolerance, was determined.

Newly fully expanded leaves of ACD plants which had been grown in an unheated glasshouse were used for calculating stomatal distribution. Transparent nail varnish was painted on the ad- and abaxial leaf surfaces and allowed to dry at room temperature. Sellotape was placed over the nail varnish and allowed to adhere to the impression. The sellotape plus impression was then removed from the leaf and viewed under a microscope to observe the imprints left by the epidermal cells. Using a 16 × 10X magnification for observation, the average number of stomata in three microscope fields for each leaf surface calculated as stomatal number per mm^2 of leaf surface, was taken as the stomata number for that genotype.

2.8. Isozyme variation

Lewis *et al.* (1980), and Humphreys and Ghesquière (1994) demonstrated that genes coding for the isozyme phosphoglucoisomerase (PGI/2) were located on three different chromosomes in tall fescue, and they constituted one homoeologous group. In the pentaploid hybrid (*Lm/Fa*) used here for anther culture, the "a" and "b" PGI/2 alleles labelled the two homologous *Lolium* chromosomes and the "a^+", "c" and "e" alleles labelled three homoeologous chromosomes carrying PGI/2 genes (see Fig. 2.1).

The different isoenzyme phenotypes for the PGI/2 locus were separated by their charge : mass ratio during starch gel electrophoresis. This involved placing the paper wick-soaked crude extract from young leaves of ACD plants (as described by Lewis *et al.*, 1980; Hayward and McAdam, 1975) towards one end of a 10% potato starch gel. Each end of the gel was covered by sponges immersed in lithium borate electrolyte buffer attached to the (+) and (-) terminals of a suitable power pack. On passing an electric charge through the gel, at a maximum current of 50 mA and a voltage of less than 300 volts, PGI/2 isozymes migrated at slightly different speeds depending on their size and charge and after 4-5 hours they occupied different positions on the gel. The gel was sliced into three sections of 2 mm thickness. The middle slice was placed in the staining substrate solution for PGI/2 and incubated at 37 °C in the dark for 20-30 min (until bands appeared). Finally the bands were read according to their positions and compared with the bands of the *Lm/Fa* pentaploid hybrid.

2.8.1. Procedures for electrophoretic separation of isozyme variants

Variants of phosphoglucoisomerase (PGI/2) isozyme loci were separated by starch gel electrophoresis.

Young actively growing and healthy leaves were selected and 2 cm pieces removed and crushed in 1 or 2 drops of "Extraction Buffer" (see below). The homogenate was absorbed onto 2 mm wide paper wicks which were inserted into a slit cut across a horizontal potato starch gel 4 cm from the cathode. 20 to 25 such wicks were accommodated on one gel.

The gels were run at 35 mA (and 200-220 volts) before removal of the wicks and 50 mA (and approximately 300 volts) afterwards. Electrophoresis was continued for about 4 hours until the borate front had migrated between 8-10 cm. All the systems migrated anodally.

Buffers and staining solutions:

1) Buffer used for homogenization of plant leaves:

TRIS - 0.605 g

2-Mercaptoethanol - 50 µl

Tris pH was adjusted to 7.2 in a final volume of 50 ml (i.e. 0.1 M TRIS).

2) TRIS/Citrate and Lithium/Borate Gel and Electrode Buffers:

(a) Boric Acid - 12.37 g - adjusted to pH 8.0 with 1 M Lithium hydroxide in a final volume of 1 litre (i.e. 0.2 M Borate).

(b) TRIS - 6.05 g - adjusted to pH 8.0 with 1 M citric acid in a final volume of 1 litre (i.e. 0.05 M TRIS).

Gel buffer:

9 parts of (b) to 1 part of (a) (usually 450 mls (b) to 50 mls (a). The gel was prepared by adding 50 g Sigma starch to 500 mls of this mixture.

Electrode buffer:

Buffer (a) - 0.2 M Borate only.

Staining solution for phosphoglucoisomerase (PGI/2):

Fructose-6-Phosphate	15 mg
N.A.D.P.	7 mg
M.T.T.	15 mg
Phenazine Methosulphate	5 mg
Glucose-6-Phosphate Dehydrogenase	20 units
1 M $MgCl_2$	1.0 ml
TRIS/HCl 0.1 M pH 7.5	100 ml

The above mixture was incubated in dark for 20 min. Gels were assayed in the dark at 37 °C for as long as required to stain the gels.

2.9. Cytological procedures

Chromosome counting of ACD plants was carried out according to the techniques which have been developed by cytologists at IGER (e.g. Morgan, 1976). Briefly, roots were produced from detached vegetative tillers on an aerated culture tank. The tips of these roots were pre-treated in distilled water at 1-2°C for 16 hours and fixed in 3:1 absolute alcohol : glacial acetic acid for

a minimum of two hours. They were then hydrolysed in 1 N HCl at 60°C for 10 minutes and stained in prewarmed Feulgen solution. Preparations of squashed root tips were made on microscope slides using 45% acetic acid solution or acetocarmine and viewed under a microscope immediately. The chromosomes of cells at metaphase were counted. The chromosome number of each genotype was taken as three cells with a consistent chromosome number.

Poor cell division and difficulties in obtaining accurate chromosome counts necessitated the use of a Flow Cytometer to determine ploidy levels in some anther culture derived plants to complete the analysis of androgenic plants. Young actively growing leaf samples free of any disease were placed in water in Eppendorf tubes and sent for analysis. Cytometer readings were carried out by Dr Marie-Claire Kerlan at INRA, Lusignan, France.

2.10. Genomic *in situ* hybridization (GISH)

Genomic *in situ* hybridization is a technique to distinguish DNA of different species. It relies on the labelling of total genomic DNA of one species with a fluorescent compound. This DNA when used as probe on chromosome preparations of a hybrid will hybridize preferentially onto chromosomes which carry the same species specific repetitive sequences.

Mitotic chromosome preparations of the two most drought (field experiment 1994, see 2.3) and cold tolerant (see 2.4) ACD plants were used as substrate for GISH to determine their chromosome constitution.

Humphreys *et al.* (1995) had determined that *F. arundinacea* was derived from two *Festuca* species, *F. pratensis* and *F. glaucescens*. The DNA of the two *Festuca* species and of *L. multiflorum* can be distinguished using GISH (see Chapter 8). Consequently, using total genomic DNA of *L. multiflorum* as a probe

on mitotic preparations of the two ACD plants, the chromosome constitution of the two stress tolerant androgenic plants was determined.

The two most cold tolerant and drought resistant anther culture derived plants (Nos. 193 and 219) were chosen for a detailed cytological study using GISH. Mitoses from root tip cells were used as substrate for *in situ* hybridization with total genomic DNA probe (TGP) from *L .multiflorum* (2x). The DNA of *F. arundinacea* was used as "blocker" to aid discrimination of *Lolium* and *Festuca* chromosomes. Additional use of *F. pratensis* and *F. glaucescens* DNA as probe by Drs. M.W. Humphreys and I. Pasakinskiene at IGER on the same two genotypes confirmed the validity of conclusions drawn from use of the *Lolium* probe.

2.10.1. Preparation of cells

Roots were produced from detached vegetative tillers on an aerated culture tank. Excised root tips were placed in ice cold water for 16 hs. and then fixed for a minimum of 2 hs. in ethanol : acetic acid (3:1). After fixation, the root tips were washed in citric acid-sodium citrate buffer (pH 4.8) and treated with an enzyme solution of 2% cellulase, 20% pectinase for 15 min at 37 ºC. Treated root tips were again rinsed in citrate buffer, transferred to 45% acetic acid for not more than 15 min, and squashed in 45% acetic acid on chromic acid cleaned slides. Slide preparations were examined under a phase contrast microscope, and those with acceptable numbers of somatic metaphase cells were frozen using a freezing aerosol spray and the cover glass removed. Preparations were stored at -20 ºC until required.

2.10.2. Preparation of the probe and *in situ* hybridization:

Total genomic DNA was extracted from young actively growing leaves of *L. multiflorum* and *F. arundinacea* (and of *F. pratensis*, and *F. glaucescens*)

according to the method of Dellaporta *et al.* (1983). The genomic probes were prepared by mechanically shearing the *Lolium* DNA to around 5 kb by submersing an Eppendorf tube containing 20 µg/200 µl for 6 min in a sonication bath. They were then labelled directly by nick translation using rhodamine-4-dUTP (Amersham). Blocking *Festuca* DNA was prepared by autoclaving for 2 min which fragmented it into approximately 200 bp.

The protocol followed for *in situ* hybridization was essentially that of Leitch *et al.*, (1994). 40 µl of hybridization mixture containing 100 µg of *Festuca* probe and up to 4 µg of blocking DNA was applied to each slide (Thomas *et al.*, 1994). All preparations were counterstained with DAPI. The preparations were mounted before examination in Vectashield antifade and viewed on a Leitz Laborlux fluorescence microscope at IGER. All photographs used in the initial phylogeny study were taken using Kodak Ektachrome 400HC slide film.

The procedures of *in situ* hybridization and interpretation of results were carried out in collaboration with Dr. Michael W. Humphreys (IGER, UK) and Dr. Izolda Pašakinskiene (Lithuanian Institute of Agriculture, Lithuania).

2.11. The cytogenetic analysis

Over all, 507 green anther culture derived (ACD) plants were established in the soil (see chapter 3). Great care was taken to discriminate genotypes on the basis of past tissue culture history and morphological characteristics and these were the bases for selection for the drought and freezing experiments (Chapter 5 and 6).

For cytogenetic analysis (Chapter 8), further selection criteria were used to discriminate between androgenic genotypes. These took into account possible somaclonal variation between plants with common or near identical genotype which became apparent in the later stages of the programme. The additional

selection criteria were difference in isozyme banding pattern, and chromosome number. Only 221 genotypes were used for cytological analysis. While the majority of the other 286 androgenic plants would have different genotype they were excluded from the cytogenetic analysis to eliminate any small possibility that plants may share the same genotype and by their inclusion skew PGI/2 segregation data.

CHAPTER THREE

ANTHER CULTURE OF AN INTERGENERIC PENTAPLOID HYBRID, *LOLIUM MULTIFLORUM* (4X) × *FESTUCA ARUNDINACEA* (6X)

3.1. Introduction

There are many species of forage grasses that are of potential use in grassland improvement. The combination of Italian ryegrass (*Lolium multiflorum*, 2n = 2x = 14) and tall fescue (*Festuca arundinacea*, 2n = 6x = 42) offers a good combination of agronomic characteristics in the *Lolium-Festuca* complex. The grasses within the *Lolium-Festuca* complex are two of the major agricultural species and present a range of complementary characters important in the development of consistently productive and persistent grass swards. Italian ryegrass provides rapid establishment and good forage quality and yield, superior in these respects to tall fescue; and tall fescue has better winter hardiness, drought tolerance and persistency than Italian ryegrass (Table 1.5) (Thomas and Humphreys, 1991). Considerable progress has been made in the production of synthetic species by artificially creating intergeneric hybrids, so as to combine the complementary characters, good nutrient value and high yield of Italian reygrass with better adaptation to environmental stresses of tall fescue.

The hybrids of the above two species are more vigorous and have either a better performance than both parents in many characters or they have a performance intermediate between the parents in some other characters. For example, Buckner *et al.* (1961) reported that some of the F_1 hybrids possessed foliage resembling ryegrass parents and all of them were superior in palatability and vigour than *F. arundinacea* Ky.31. Dijkstra and Devos (1975) compared seedlings of allopolyploid families from *L. multiflorum* × *F. arundinacea* hybrids with varieties of the two parental species at different stages in a heated and an unheated glasshouse, and in the field. They found that the early seedling growth in the hybrid families was in most cases significantly better than for two tall fescue varieties. This applied to height, number of tillers, leaf width and yield of green matter. The pentaploid hybrid used here (see chapter 2) was vigorous, taller, and had more tillers and dry matter yield than *L. multiflorum* and *F. arundinacea* genotypes used as controls.

The F_1 (2n = 4x = 28) of *L. multiflorum* (2x) and *F. arundinacea* (6x) is sterile. The restoration of fertility of such a hybrid was possible by doubling the chromosome number. The amphiploid, which can be produced by doubling the chromosomes number of the parents prior to crossing, has 56 chromosomes. Lewis (1982) reported a high level of bivalent pairing in such octaploid plants and he assumed this to be largely preferential. He did not find sufficient preferential pairing in the synthetic allotetraploids between *F. pratensis* and *L. multiflorum* or *L. perenne* species to prevent their subsequent loss of stability. In this instance the situation was aggravated by considerable chromosome loss (Lewis, 1966). Amphiploid hybrids, with complete genomes from reygrass and *F. pratensis*, unfortunately expressed both advantageous and disadvantageous parent traits (Humphreys, 1994). Efforts to breed hybrid cultivars have thus far been frustrated by genetic instability of amphiploids and problems of seed production and the predominant weakness of the amphiploid i.e. slow germination and poor initial seedling growth (Thomas and Humphreys, 1991).

In another approach exploitation of hybrids between *L. multiflorum* and *F. arundinacea* through the controlled introgression of genes or gene blocks rather than genome incorporation was attempted. Controlled introgression aimed to introduce the smallest possible segment of the alien genome into the cultivated species to avoid introducing any further genetic material that could be detrimental to the performance of the recipient (Thomas, 1993). Lewis (1970) did attempt to transfer a limited number of *F. arundinacea* characteristics, such as spring growth, winter hardiness and persistency into a tetraploid *Lolium* background by means of a backcross programme involving the amphiploid but the complex nature of these characters, and the absence of any suitable markers, made effective screening of each generation impossible and the programme was discontinued after three generations. A 42 chromosome (Kenhy) tall fescue-like cultivar was stabilized with certain Italian reygrass characteristics by back-crossing the amphiploid *L. multiflorum* × *F. arundinacea* (2n = 8x = 56) onto *F. arundinacea* by Buckner *et al.* (1977).

Humphreys (1989) by using phosphoglucoisomerase (PGI/2) as a genetic marker, showed the possibility of transferring genes from *F. arundinacea* into diploid *L. multiflorum* using the *Lm/Fa* pentaploid hybrid (see chapter 2) as a male parent after a second cycle of back-crossing. He then improved drought resistance in some of these introgression plants equivalent to that of *F. arundinacea* (Humphreys and Thomas, 1993). It was also demonstrated that this improved drought resistance was due to the transfer of genetic material from *Festuca* into *Lolium*. The frequency of recombinants reported by Humphreys (1989) and Humphreys and Thomas (1993) was low (3%), and large populations of BC_2 derivatives were required to detect rare recombinants.

An alternative approach to select for drought tolerance was to seek exploitation of the combination of Italian ryegrass and tall fescue characters by the production of gametophytic progenies from the *Lm/Fa* pentaploid hybrid. The aim of the current study, was to explore anther culture as a means of improving the chance of intergeneric recombination by following the exchange of genetic material in the male gametes (microspores) of the *Lm/Fa* pentaploid hybrid (see chapter 2). The chromosome complement of these anther culture derived (ACD) plants could be doubled either spontaneously or by using cholchicine treatment to produce stable homozygous plants. It was assumed that a number of possible combinations between and within the genetic material of both genomes could occur at meiosis of the microspores, including the expression of recessive genes, which was extremely difficult by conventional plant breeding methods.

3.2. Materials and methods

3.2.1. Plant materials (Source of anthers)

Anthers used in culture were all derived from a single genotype of a *L. multiflorum* × *F. arundinacea* 2n = 5x = 35 hybrid *Lm/Fa* (see chapter 2). The pentaploid hybrid was separated into 121 clonal parts to produce sufficient panicles for use in anther culture. All plants were initially established in a heated glasshouse and were subsequently transferred to an unheated glasshouse for vernalization for 8 weeks.

Heading time was extended by maintaing plants in a range of environmental and fertilizer treatments, summarized in Table 3.1.

Table 3.1: Different environments for *Lm/Fa* pentaploid hybrid following vernalization. (E = environment)

E1	Unheated glasshouse.
E2	Unheated glasshouse with additional nitrogen fertilizer. Application of 0.12 g of high rate nitrogen fertilizer (NPK, 36:6:6) per pot on 31.3.1993 and 3.5.1993.
E3	Heated glasshouse 23-25°C photoperiod of 20 hours.
E4	Outside of glasshouse
E5	Cool-room (9.5-13°C) and short day length (8 hours light) for 95 days from 6.4.1993 until 9.7.1993.
E6	Cool-room (9.5-13°C) and short day length (8 hours light) for 95 days from 6.4.1993 until 9.7.1993 and with extra nitrogen fertilizer as above.

3.2.2. Anther culture medium

For anther culture a modified MS (Murashige and Skoog, 1962) medium was used with minor changes (Ward, 1993) (Table 3.2).

Table 3.2: Embryo induction medium

Medium component	Final concentration ($mg\ l^{-1}$)	Volume of stock solution added for 1 litre (ml)
Minerals		100
Thiamine	1	5
FeNaEDTA (Iron)	36.75	5
Kinetin	0.5	5
2,4-D	0.5	15

Maltose was added to give a 9% solution in the final medium.

The medium above was made up to 1 litre, the pH adjusted to 6.0 then the medium was equally divided into 500 ml, each containing 2.0 g of Gel-rite (4.0 g/ litre). The flasks were covered with double layer of foil and autoclaved under pressure 1 kg/cm^2 (15 lbs/ in^2) at 121°C temperature for at least 15 minutes. When the medium had cooled sufficiently for handling, it was poured into 9 cm Petri dishes (about 18-20 ml per Petri dish) in a sterile microflow air cabinet. The medium was allowed to set in the Petri dishes which were then wrapped in cling film or in sterile plastic bags and stored in a cold room.

The macro mineral stock solution once prepared was stored in 100 mls bottles in a cold room at +4°C (Table 3.3).

Table 3.3. Macro mineral stock solution (for embryo induction medium)

Medium component	Concentration ($g\ l^{-1}$)
KNO_3	10.00
$(NH_4)_2\ SO_4$	1.00
KH_2PO_4	2.00
$Ca(NO_3)_2\ 4H_2O$	1.00
$MgSO_4\ 7H_2O$	1.25
KCl	0.35

The iron and vitamin stocks were prepared as 5 ml volumes and stored prior to use at -20°C. Unlike Ward (1993), the iron and vitamin stocks solutions were kept separate (Table 3.4).

Kinetin stock solution was prepared by dissolving 10 mg kinetin with 1 ml NaOH 1M in 100 mls of distilled water and 2,4-D stock solution has dissolving 10 mg 2,4-D with 2 mls of 100% ethanol then diluting with distilled water up to 100 mls. The kinetin and 2,4-D stock solutions were stored at +4°C (Table 3.4).

Table 3.4. Micronutrient stock solutions (for embryo induction and regeneration media).

Medium component	Concentration ($mg\ l^{-1}$)
Thiamine HCl	200
FeNa EDTA	7340
Kinetin (6-furylaminopurine)	100
2,4-D	100

The regeneration medium (Table 3.5) was made up to 1 litre of water, the pH was adjusted to 6.0 and the medium divided equally into 500 ml each containing 4.0 g Agar (8.0 g/litre) or 1.5 g of gel rite (3.0 g/litre). For long term storage, agar was used since gel-rite is known to induce mutations. The flasks were then covered with a double layer of foil and autoclaved as before. When the medium had cooled sufficiently, it was poured either into 9 cm Petri dishes (about 18-20 mls per Petri dish) for embryo induction and regeneration, or into 30 mls plastic Universal container vessels (5-8 mls per Universal) for growth of individual plantlets. The medium was allowed to set in the Petri dishes or Universals. The Petri dishes were wrapped in cling film and both they and the Universal container vessels stored in sterile plastic bags in a cold room at +4°C.

Table 3.5. Regeneration medium for callus

Medium component	Concentration ($g\ l^{-1}$)
MS (Murashige and Skoog) basal salt mixture	4.3
Sucrose	30
Kinetin	0.002

Table 3. 6. Murashige and Skoog (MS)

Medium component	Concentration ($mg\ l^{-1}$)
NH_4NO_3	1650.000
H_3BO_3	6.200
$CaCl_2$	332.200
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
$C_6H_6O_8Na_2$	37.260
$FeSO_4.8H_2O$	27.800e
$MnSO_4$	180.700
$MnSO_4.H_2O$	16.900
KI	0.830
KNO_3	1900.000
KH_2PO_4	170.000
$Na_2MoO_4.2H_2O$	0.250
$ZnSO_4.8H_2O$	8.600

The Murashige and Skoog medium is extremely hygroscopic and was protected from atmospheric moisture. In its anhydrous form 4.3 g of powder per 1 litre medium was used. Normally 4.71 g of MS powder was dissolved in 1 litre of distilled water. The volume of stock solution of Murashige and Skoog (MS) basal salt mixture (SIGMA) required for 1 litre of medium is presented in Table 3.6.

3.2.3. Anther culture procedure

3.2.3.1. Stage of pollen development

The developmental stage of the microspores at the time of anther excision is one of the major factors for optimum induction of anther cultures. Although embryos can be obtained from almost all microspore stages, including tetrads and almost mature pollen (He and Ouyang, 1984), an optimum stage does exist. As soon as starch deposition begins, no saprophytic development and subsequently no macroscopic structure formation (i.e., calli and embryoids) will occur (Wenzel and Foroughi-Wehr, 1984). Depending on species, this stage can be before, during or after the first mitosis of the microspore nucleus. For the cereals the most responsive stage is the mid to late uninucleate stage (Figure 3.1 stages "e" to "i"), when the nucleus of the microspore is migrating from its position near the pore to the opposite side of the cell where mitosis occurs (Wenzel and Forughi-Wehr, 1984 and Olesen, 1987).

Since the developmental stage of the microspore is crucial for the optimum responses in the culture, the pollen stage in the anther was determined microscopically using acetocarmine squashes of 1 anther per floret before anther culture. Healthy panicles of the pentaploid hybrid *Lm/Fa* with the pollen grain of the oldest florets at the uninucleate well vacuolated stage up to pre-mitotic stage (Figure 3.1 stages "e" to "i") were excised. The panicles were cut below the lower nodes and stood in a beaker of tap water.

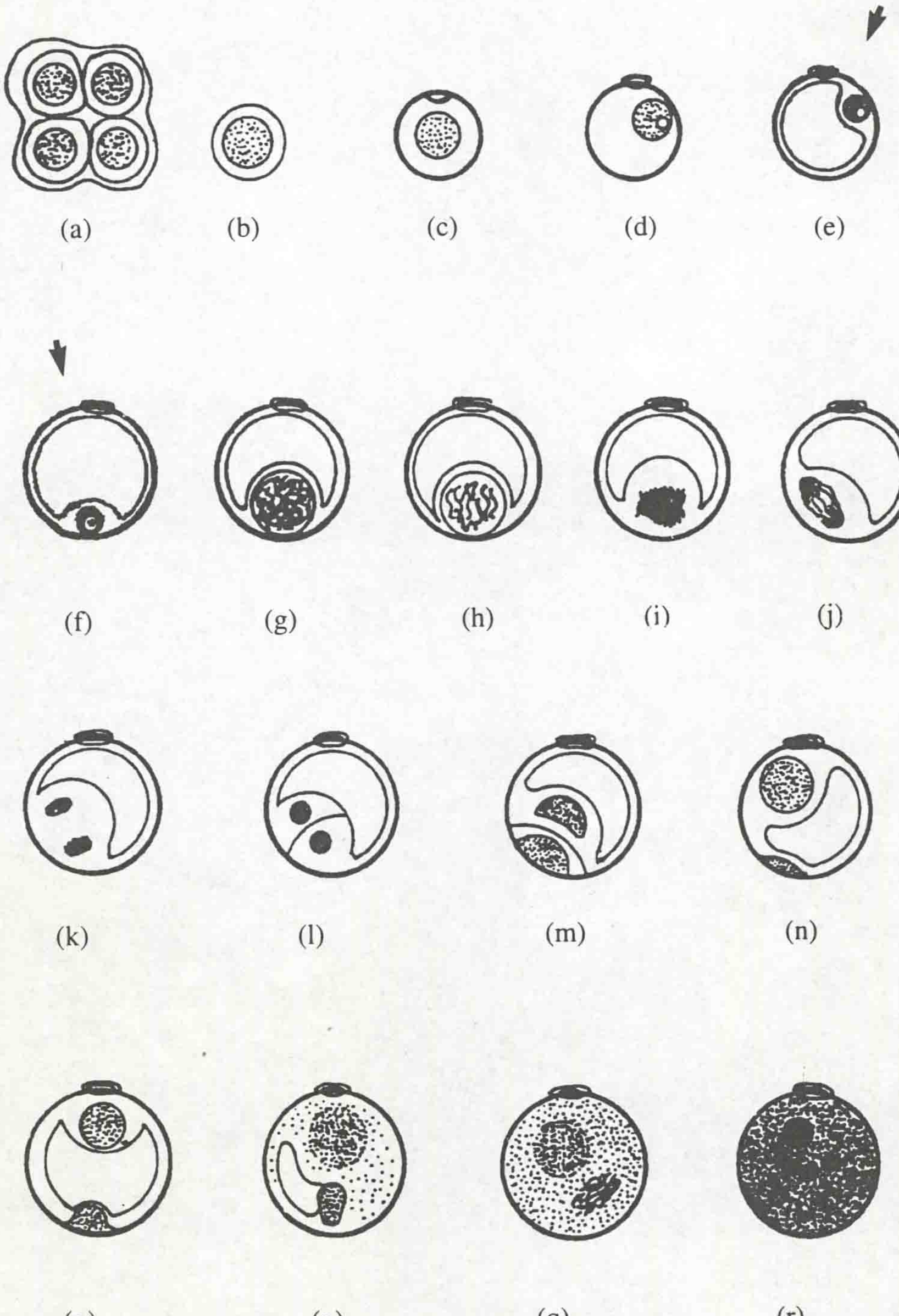


Figure 3.1. Pollen grain developmental stages. The stages most suitable for anther culture are indicated (e, f) (Sue Dalton, pers. comm.).

3.2.3.2. Pre-treatment of panicles

In order to maximise opportunities for successful anther culture, various pre-treatments were attempted. A cold pre-treatment is more or less accepted by researchers as having a beneficial affect on microspore induction and response in anther culture. A cold pre treatment was not routinely used in the current study but in some cases panicles were stored at 4-6°C in darkness for up to five days prior to culture.

3.2.3.3. Culturing the anthers

Panicles were sterilized by a brief rinse (approximately 15 seconds) in 70% ethanol before being immersed in 0.1% mercuric chloride for eight minutes. They were subsequently rinsed three times in sterile distilled water. The spikelets as mature as those spikelets examined were removed and placed in a sterile Petri dish. Anthers at the correct stage from the two oldest florets of the spiklets were excised by forceps, then placed onto the embryo induction medium in 9 cm plastic Petri dishes. When each dish contained 22-116 anthers, it was sealed with parafilm, labelled and incubated in the growth room at approximately 25°C in continuous florescent light (1500 W/m^2). The culture conditions were contrary to those used for incubating cereal anthers (Clapham, 1977), wheat (Luckett *et al.*, 1991), maize (Barloy and Beckert, 1993), *Lolium* and *Festuca* species and their hybrids (Ward, 1993) and tall fescue anthers (Kasparbauer *et al.,* 1980) which were all maintained in the dark.

The cultures were examined after a few days for fungal contamination. Regions of contamination were removed and badly contaminated Petri dishes discarded. Most contamination was found in surface sterilized glass Petri dishes rather than the presterilized plastic ones. Only a few anthers were damaged in the

process of transfer to culture media, but development sometimes occurred even in damaged anthers.

3.2.3.4. Regeneration of calluses and plantlets

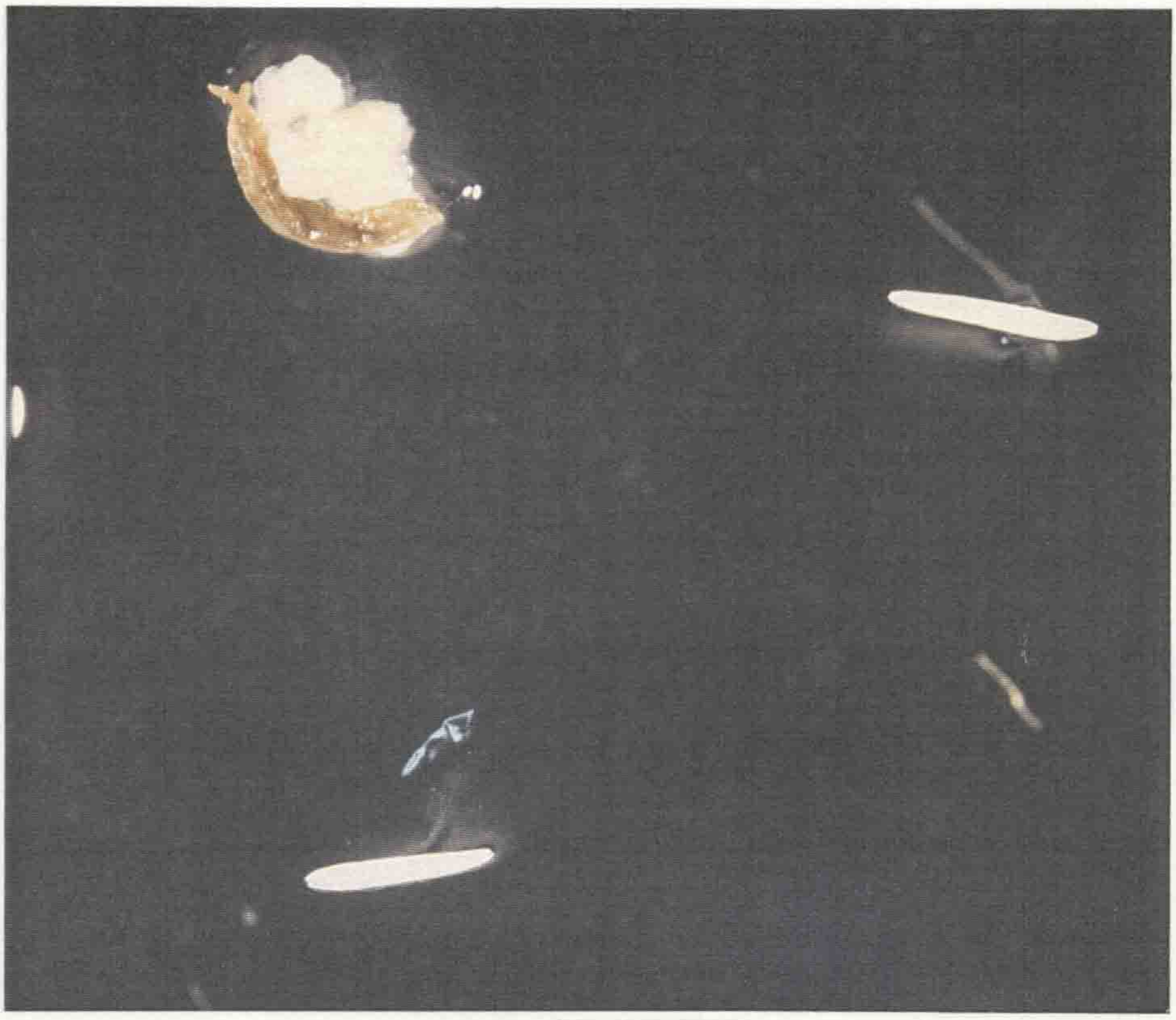
After a few days culture most anthers turned from green to dark brown, Figure 3.2. Anthers which turned white after a few days did not develop. Callus developed out of culture responsive anthers after 4-6 weeks (Figure 3.3). The anthers of the pentaploid *L. multiflorum* × *F. arundinacea* hybrid like other forage grass anthers remained intact in culture unlike the cereals where the anthers open within a few days. At a very early stage after placing the anthers onto the induction medium, callus appeared as small transparent blisters on the anther surface which were visible by magnifying glass or even by eye.

Following the initial development which occurred within 4-6 weeks, anthers were inspected every few days and the anthers plus callus were transferred to regeneration medium (also in Petri dishes). If more than one embryoid was seen to develop within a single callus, the embryos were carefully separated prior to transfer to a regeneration medium.

The germinated green or albino plantlets (Figure 3.4A) were individually transferred to sterile 30 ml plastic universal containers (Figure 3.4B) which contained about 5-8 mls of regeneration medium for further growth and kept in the same regeneration environment (approximately 25°C in continuous florescent light, 1500 W/m^2).

Figure 3.2: Anthers after 1-2 weeks in culture. Anthers which were likely to develop callus turned brown. No microspore development was observed in anthers which turned to white or colourless.

Figure 3.3: Production of callus from developing anthers (brown anthers) on embryo induction medium.



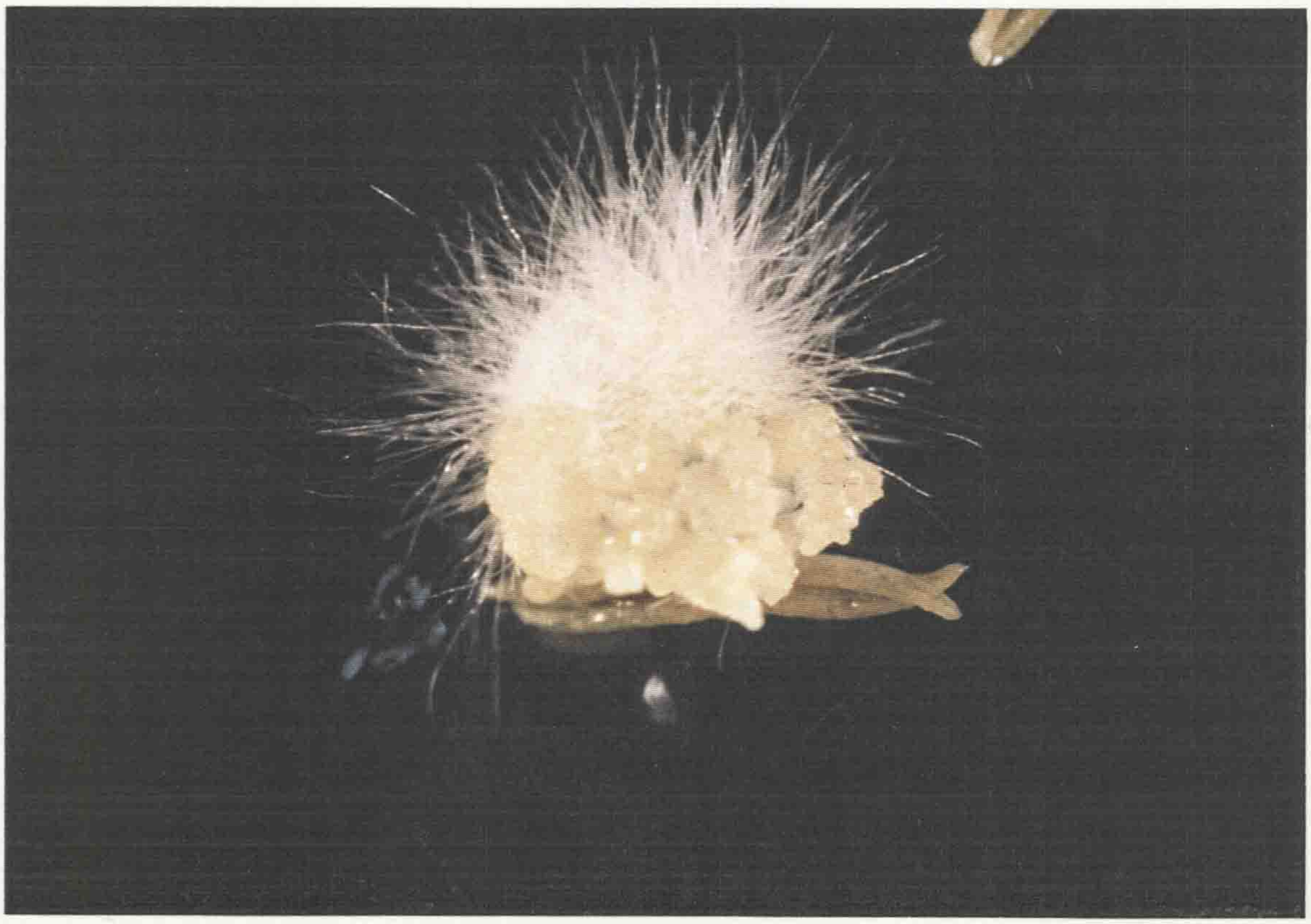


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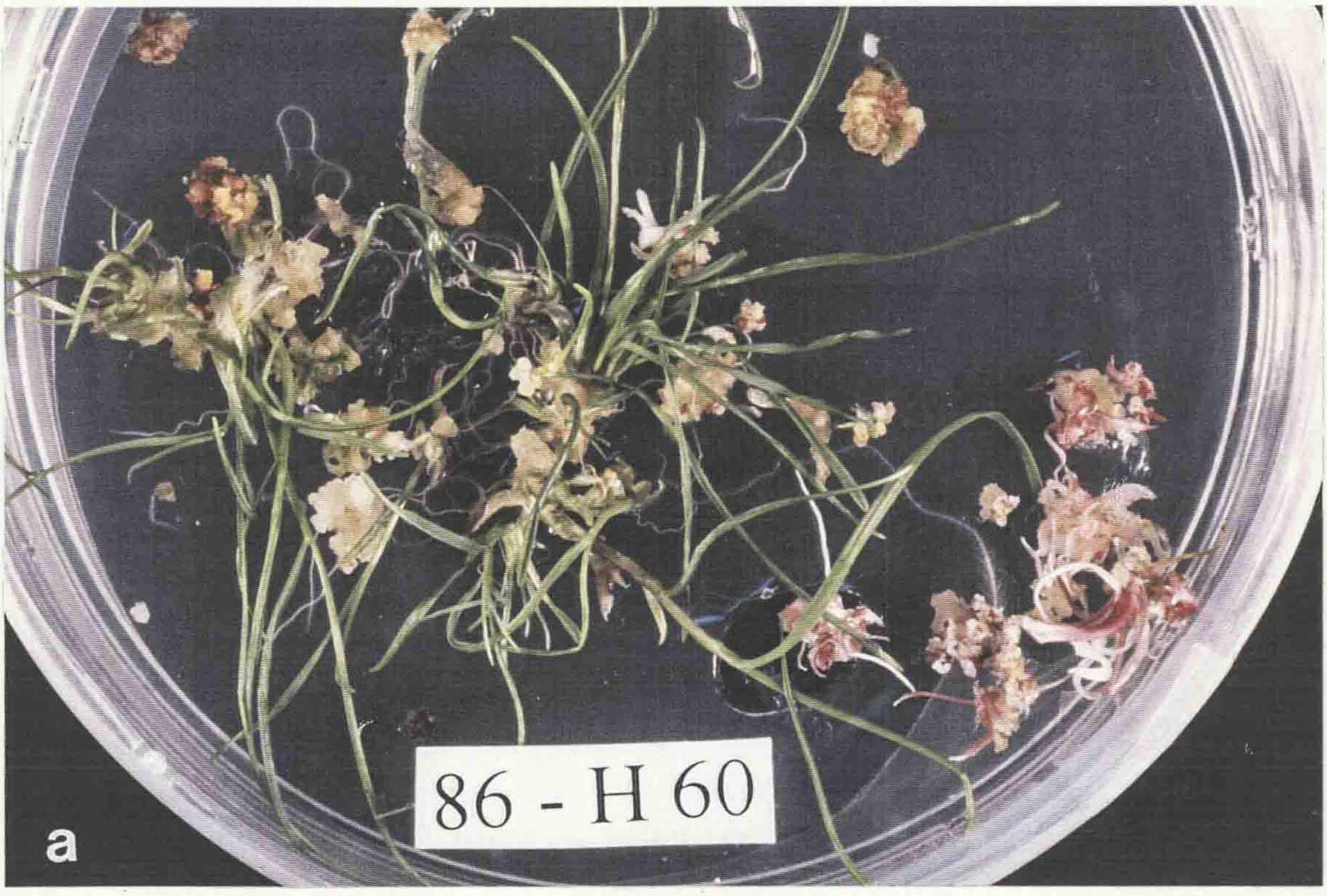
Figure 3.4: A - Plantlets (green and albino) developing from callus subculture over regeneration medium. B - Isolated plantlet in 30 ml universal sterile tubes containing regeneration medium.



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3.2.3.5. Planting regenerated plantlets in soil

Green plantlets when 3-4 cm high were carefully removed from the universal containers and transferred to soil. The size of shoots and roots of plantlets varied. While some plantlets were weak with a small root or even rootless others were quite robust with large strong roots and could be transferred directly into soil from the Petri dishes. If tillers in any plantlets appeared morphologically dissimilar, they were split and transferred separately into soil. Any remaining medium on the roots of plantlets was washed with sterile distilled water to remove agar and prevent contamination. Anther culture derived plantlets were planted in small pots (6 cm) containing sterile soil in a warm glasshouse at 18-25°C.

Young plantlets were maintained in a humid environment under inverted clear plastic containers. Regenerants were subsequently transferred to 13 cm pots in potting compost and left in an unheated glasshouse.

3.2.3. Analysis of data

Analysis of variance was used to determine the effects of different variables on the success of anther culture. These variables were (1) the glasshouse environments and fertilizer application (2) cold treatment factor up to flowering prior to culture, (3) height of stem and (4) pollen stage. Analyses were confined to panicles which yielded plantlets. Analyses of variance (GLM type III) were calculated using SAS (The Statistical Analysis System, 1990). Significant differences amongst treatment means were compared by Ryan-Einot-Gabriel-Welsch multiple F-test and Boniferroni (Dunn) T-tests.

3.4. Results and discussion

Successful culture of anthers is very dependent on genotype (Guha-Mukherjee, 1973; Bajaj, 1976; Taylor and Veilleux, 1992 and Luckett and Smithard, 1992), conformation of callus induction and regeneration media, condition of the donor plant and pollen grain, and the stage of development of the anthers when first placed *in vitro*.

The results of the anther culture of the pentaploid hybrid *L. multiflorum* × *F. arundinacea* (2n = 5x = 35) are summarised in Table 3.7. From a total of 33 panicles used, only anthers from one panicle were discarded because of a high fungal contamination and anthers of five more panicles did not show any response in culture. The over all mean as demonstrated in Figure 3.5 showed that from 100 cultured anthers, 26 anthers showed signs of development and 74 failed to develop of which 6 died during the first two weeks in culture, i.e. their colour turned from green to white instead of turning to brown. The number of calli developed from 100 cultured anthers was 133 or alternatively more than five calli developed from each anther in culture. A total of 2349 plantlets were derived from 3326 cultured anthers (Table 3.7) i.e. 70.6 plantlets per 100 cultured anthers. 57% of the plantlets were green and 43% albino. The albino plants were discarded because they would not survive out of artificial media and be unable to synthesize carbohydrate for their metabolism. Less than half of the green plantlets could be transferred into sterile soil. A likely explanation for this high mortality during establishment was chromosome imbalance resulting from aneuploidy, chromosomal abnormality, or chromosomal deletion and duplication. Stresses arising from splitting up and sub-culturing the plantlets was another possible reason for their high mortality. Once transferred to sterilzed soil, 507 plantlets (79.50%) developed (or 21.6 ACD plants from 100 cultured anthers).

Table 3.7. Anther culture of the *Lm/Fa* pentaploi hybrid.

	Number	% production
Panicles used	33	-
Cultured anthers	3326	100.0
Responsive anthers	865	26.0
Non responsive anthers	2293	68.9
Anthers dead after 2-3 weeks	168	5.1
Callus cultures	4419	132.9
Total plantlets	2349	70.6
Albino plantlets	1010	30.6
Green plantlets	1339	40.3
Plantlets in to sterile soil	638	19.2
Established ACD plants	507	15.2

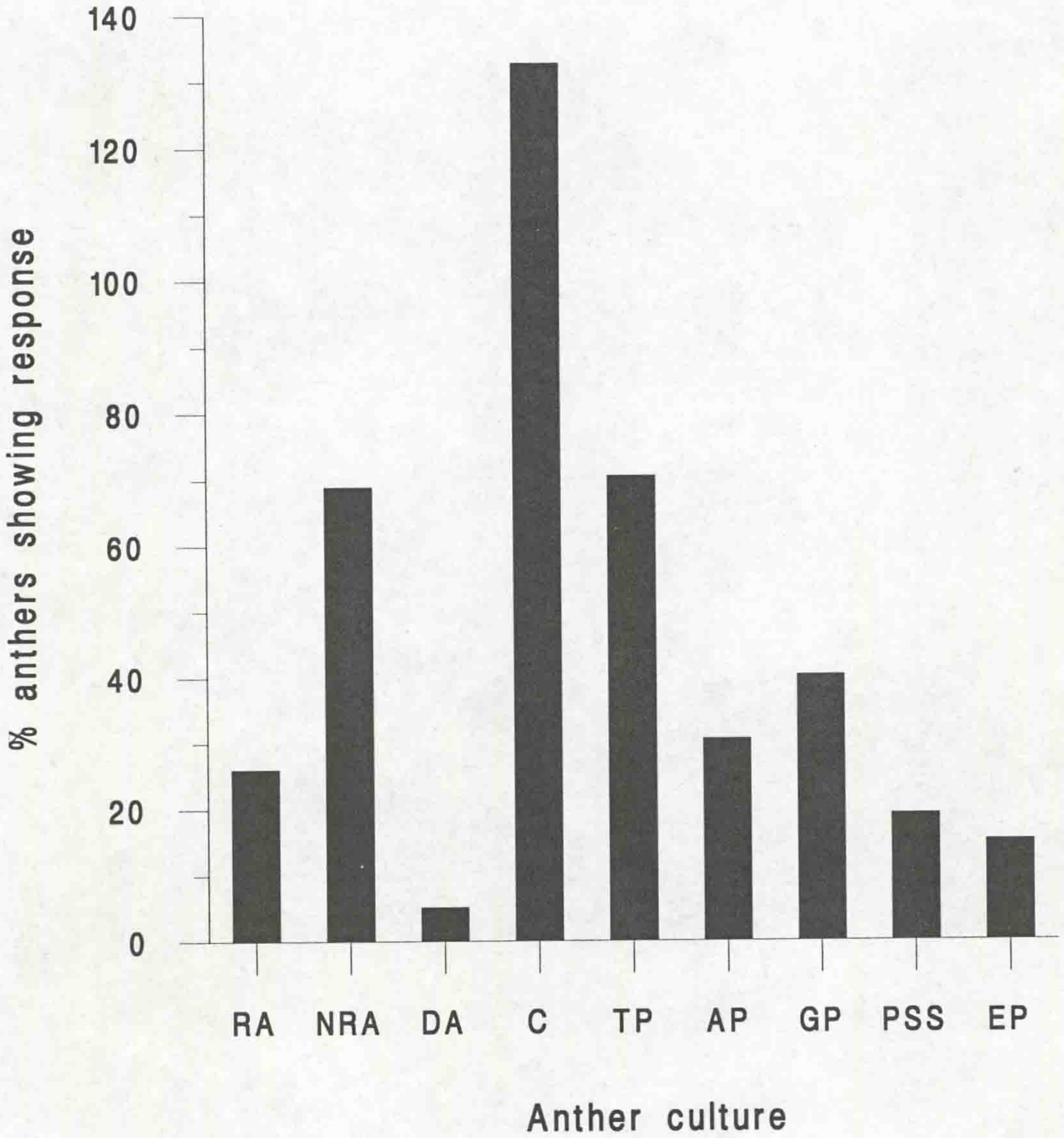

Figure 3.5. The responses of anther culture and its production from 100 cultured anthers of *Lm/Fa* pentaploid hybrid. RA = responding anthers, NRA = non responding anthers, DA = dead anthers, C = callus and sub-callus, TP = developed plantlets, AP = albino plantlets, GP = green plantlets, PSS = plantlets transferred to sterile soil and EP = established ACD plants.

The environmental conditions for plant growth i.e. a cool-room, a heated glasshouse, an unheated glasshouse, outside the glasshouse and the level of fertilizer application determined the subsequent vigour of the *Lm/Fa* pentaploid hybrid and flowering time.

Most of the cultured anthers (69.2%) were taken from plants grown in an unheated glasshouse (i.e. 40.0% from plants with no fertiliser (E1) and 29.2% from plants treated with fertiliser (E2)). 14.7% were from plants grown in a heated glasshouse (E3), 8.1% from plants grown outside the glasshouse (E4) and 14.0% were from plants grown in a cool-room (i.e. 11.9% were from plants with no fertiliser (E5) and 2.1% from plants with fertiliser (E6)), Table 3.8.

The effects of different environments (see Table 3.1) on aspects of anther culture production are presented in Table 3.8 for (i) anther response, (ii) callus initiation from anthers and also subcultured callus, (iii) total ACD plantlets, (iv) albino ACD plantlets, (v) total green ACD plantlets, (vi) green ACD plantlets transferred to sterile soil and (vii) established ACD plants in the soil. Figure 3.6 showed that there were some treatment affects on anther culture production. In general plants grown in an unheated glasshouse (E1) performed the best in terms of anther response, production of green plantlets and soil establishment. The next most successful treatment was from hybrids kept in a cool room for 95 days (E5), plants kept out of doors (E4), and plants kept in unheated glasshouse with extra fertiliser (E2). The least successful treatment arose where plants were kept in a heated glasshouse (E3). Thus plants to be used in anther culture should be kept in unheated glasshouse conditions rather than under heated glasshouse conditions. If it is necessary to extend the flowering time to increase time for culturing anthers, it is better to keep plants in a cool room with a short day length (8 hours) instead of an application of nitrogen fertilizer.

The height of the tiller was considered as one measure of plant vigour. The heights of the tiller of the panicle used in anther culture were grouped in three groups. Most of the excised tillers (61.4%) had heights between 100 - 150 cm, 22.3 % of tillers were shorter than 100 cm and the rest 16.3% of the tillers were taller than 150 cm.

Table 3.8. Effect of condition of growth on androgenesis from a *Lm/Fa* pentaploid hybrid. Numbers in parentheses are the percentage of anther culture production per 100 cultured anthers.

E	CA	RA	C	TP	AP	GP	PSS	EL
1	1092 40.0%	418 (38.3)	1800 (164.8)	1218 (111.5)	507 (46.4)	711 (65.1)	351 (32.1)	264 (24.2)
2	940 29.2%	174 (18.5)	1058 (112.6)	447 (47.6)	168 (17.9)	279 (29.7)	120 (12.8)	62 (6.6)
3	474 14.7%	48 (10.1)	552 (116.5)	106 (22.4)	33 (7.0)	73 (15.4)	47 (9.9)	57 (12.0)
4	259 8.1%	72 (27.8)	472 (182.2)	133 (51.4)	31 (12.0)	101 (39.0)	39 (15.1)	30 (11.6)
5	383 11.9%	126 (32.9)	533 (139.2)	444 (115.9)	271 (70.8)	173 (45.2)	81 (21.2)	64 (16.7)
6	68 2.1%	3 (4.4)	4 (5.9)	2 (2.9)	0 (0.0)	2 (2.9)	0 (0.0)	0 (0.0)
Total	3216 100.0 %	841 (26.2)	4419 (137.4)	2350 (73.1)	1010 (31.4)	1339 (41.6)	638 (19.8)	477 (14.8)

E: pretreatment of parent *Lm/Fa* hybrid prior to culture(see Table 3.1), CA: cultured anthers, RA: responsive anthers, C: callus and subcultured callus, TP: total developed plantlets (green or albino), AP: albino plantlets, GP: green plantlets, PSS: green plantlets transferred to sterile soil, EL: ACD plants established in normal soil.

Figure 3.6. Effect of *Lm/Fa* pentaploid hybrid growth condition on anther culture. E = environment (see Table 3.1), RA = responding anthers, C = callus and sub-caltured callus, TP = developed plantlets, AP = albino plantlets, GP = green plantlets, PSS = plantlets transferred to sterile soil and EP = established ACD plants.

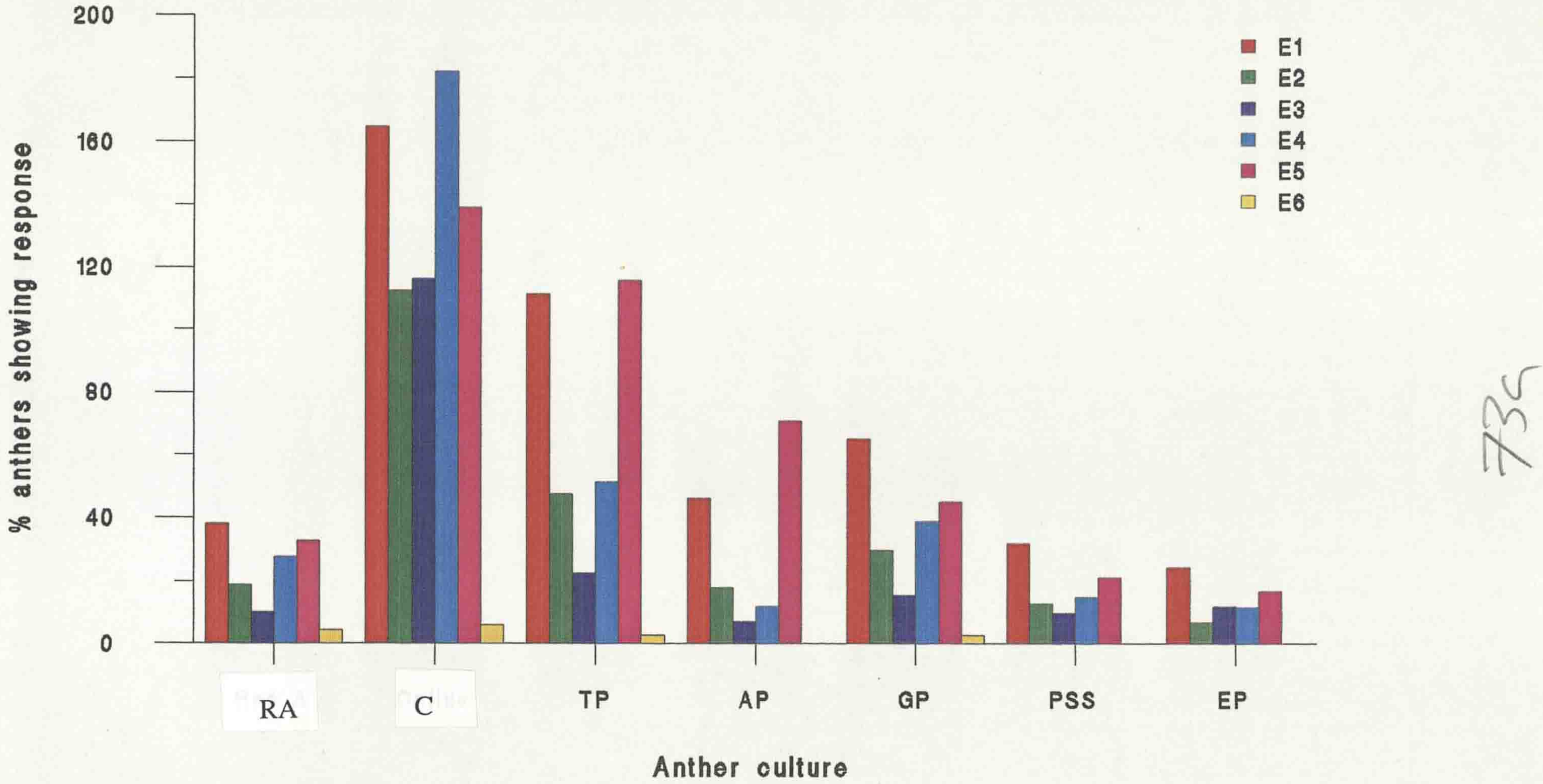
200
160
120
80
40
0
% anthers showing response
E1
E2
E3
E4
E5
E6
RA
C
TP
AP
GP
PSS
EP
Anther culture

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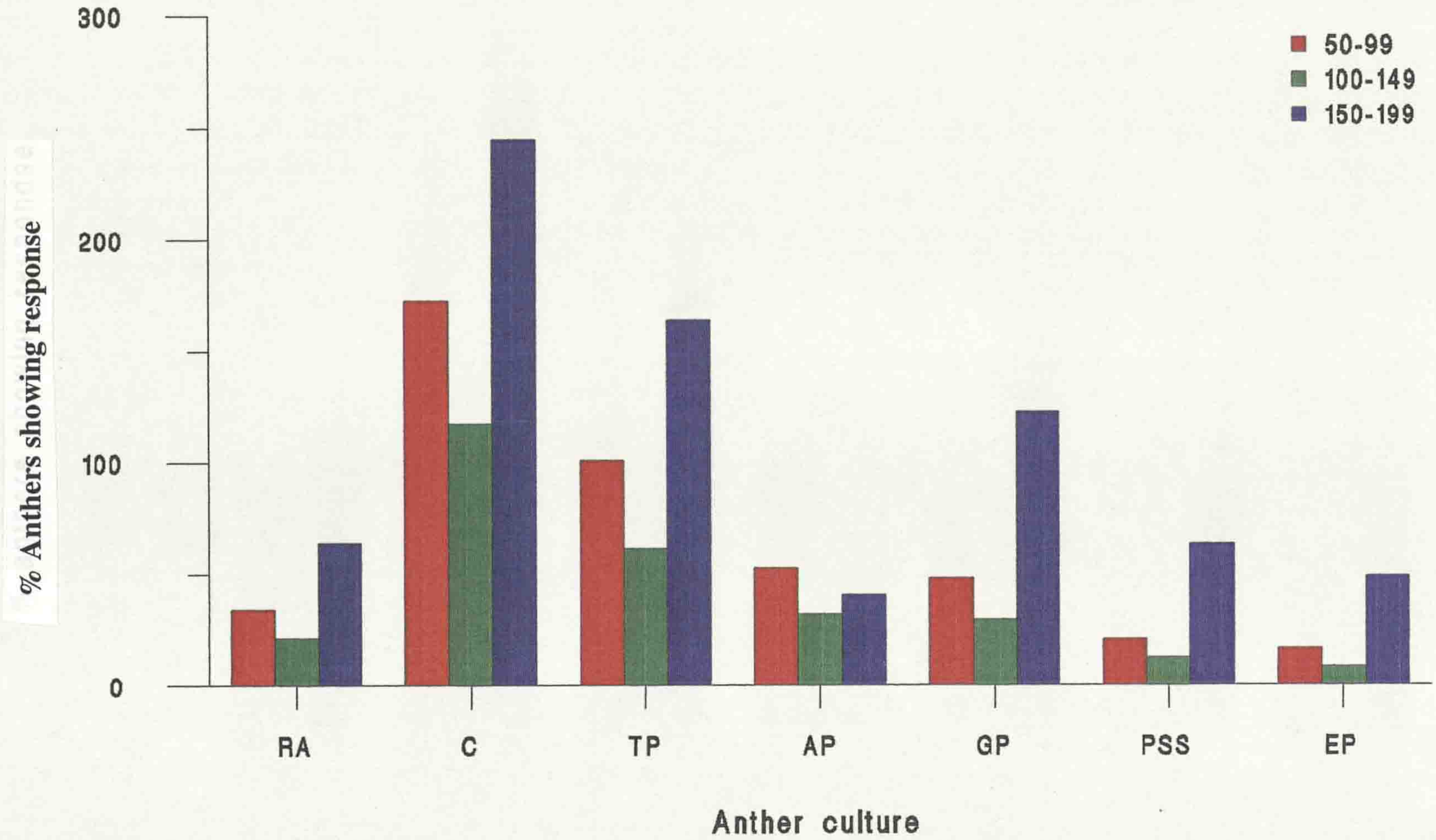
Table 3.9. Effect of plant height of *Lm*/*Fa* pentaploid hybrid on anther culture. Numbers presented in parentheses are the percentage anther culture production per 100 cultured anthers.

PH	CA	RA	C	TP	AP	GP	PSS	EL
50-99	568	195	981	574	300	274	120	94
	22.3%	(34.3)	(172.7)	(101.1)	(52.8)	(48.2)	(21.1)	(16.6)
100-	1563	337	1836	966	504	462	195	134
149	61.4%	(21.6)	(117.5)	(61.8)	(32.2)	(29.6)	(12.5)	(8.6)
150-	416	267	1020	682	171	511	266	204
199	16.3%	(64.2)	(245.2)	(163.9)	(41.1)	(122.8)	(63.9)	(49.0)
Total	2547	799	38.37	2222	975	1247	561	432
	100.0%	(31.4)	(150.7)	(87.2)	(38.3)	(49.0)	(22.0)	(17.0)

PH: height of anther donor tiller (cm), CA: cultured anthers, RA: responsive anthers, C: callus and subcultured callus, TP: total developed plantlets (green or albino), AP: albino plantlets, GP: green plantlets, PSS: green plantlets transferred to sterile soil, EL: ACD plants established in normal soil.

Figure 3.7. Effect of anther donor plant height on anther culture. RA = responding anthers, C = callus and sub-callus, TP = developed plantlets, AP = albino plantlets, GP = green plantlets, PSS = plantlets transferred to sterile soil and EP = established ACD plants.


300
200
100
0
% Anthers showing response
50-99
100-149
150-199
RA
C
TP
AP
GP
PSS
EP
Anther culture

The effects of height of the tiller of panicle (cm) on aspects of anther culture production are presented in Table 3.9. Data from Figure 3.7 suggested that anther culture production, except for the albino ACD plantlets, was highest in panicles with a height over 150 cm. Panicles from the shortest tillers yielded more albino plantlets than other groups. The overall best anther culture results were obtained from panicles on the tallest tillers. The next best anther culture results were obtained from the shortest tillers with the intermediate height class giving the poorest response to anther culture.

Since the developmental stages of the microspores is crucial for the optimum culture response, the aim was to have anthers with microspores at mid to late uninucleate stage. Because the categories of the pollen stages "e", "f", "g", "h", and "i" (see Figure 3.1) of the excised panicles were very close and because variation in anther maturity occurred within a panicle and even anthers of one flower, pollen stage had within these limits showed no significant effect on anther culture production. However other authors (Wenzel and Forughi-Wehr 1984 and Olesen 1987) have stated their the best pollen stage for anther culture of cereals and forage grasses is pollen grains at the mid to late uninucleate stage. Anthers of the *Lm/Fa* hybrid at this stage ("e" to "f", Figure 3.1) did show a good response to anther culture.

The main reason for pre-treatment of the panicles, i.e. keeping the panicles in a refrigerator at +4 to +6°C and darkness, in this study was to make the duration of the storage time more flexible. Thus most of the anthers (74.8%) were cultured on the same day as the panicle was removed from the plant, and the panicle of the rest of the anthers (25.2%) were left in a beaker of water and maintained at +4 to +6° C in darkness for one to five days (Table 3.10).

The anthers cultured from freshly cut panicles with no pre-treatments were the most responsive in culture (Figure 3.8). The best response *in vitro* also led to most green ACD plantlets and subsequently, established ACD plants. The other treatments showed only a small response with only little differences in numbers of

established ACD plants in soil. Thus for the best anther culture response for this media forage grass, anthers should be cultured on the same day. However success was not greatly reduced if panicles were stored for some days.

Table 3.10. Effect of pre-treatment on anther culture of *Lm/Fa* pentaploid hybrid. Numbers presented in parentheses are the percentage anther culture production per 100 cultured anthers.

PT	CA	RA	C	TP	AP	GP	PSS	EL
0	24.06	757	3552	2147	937	1210	563	430
	74.8%	(31.5)	(147.6)	(89.2)	(38.9)	(50.3)	(23.4)	(17.9)
1-5	810	180	867	202	73	129	75	77
	25.2%	(22.2)	(107.0)	(24.9)	(9.0)	(15.9)	(9.3)	(9.5)
Total	3216	937	4419	2349	1010	1339	638	50.7
	100.0%	(29.1)	(137.4)	(73.0)	(31.4)	(41.6)	(19.8)	(15.8)

PT: storage of panicle at +4°C and darkness prior to culturing the anthers as a pre-treatment (days), CA: cultured anthers, RA: responsive anthers, C: callus and subcultured callus, TP: total developed plantlets (green or albino), AP: albino plantlets, GP: green plantlets, PSS: green plantlets transferred to sterile soil, EL: ACD plants established in normal soil.

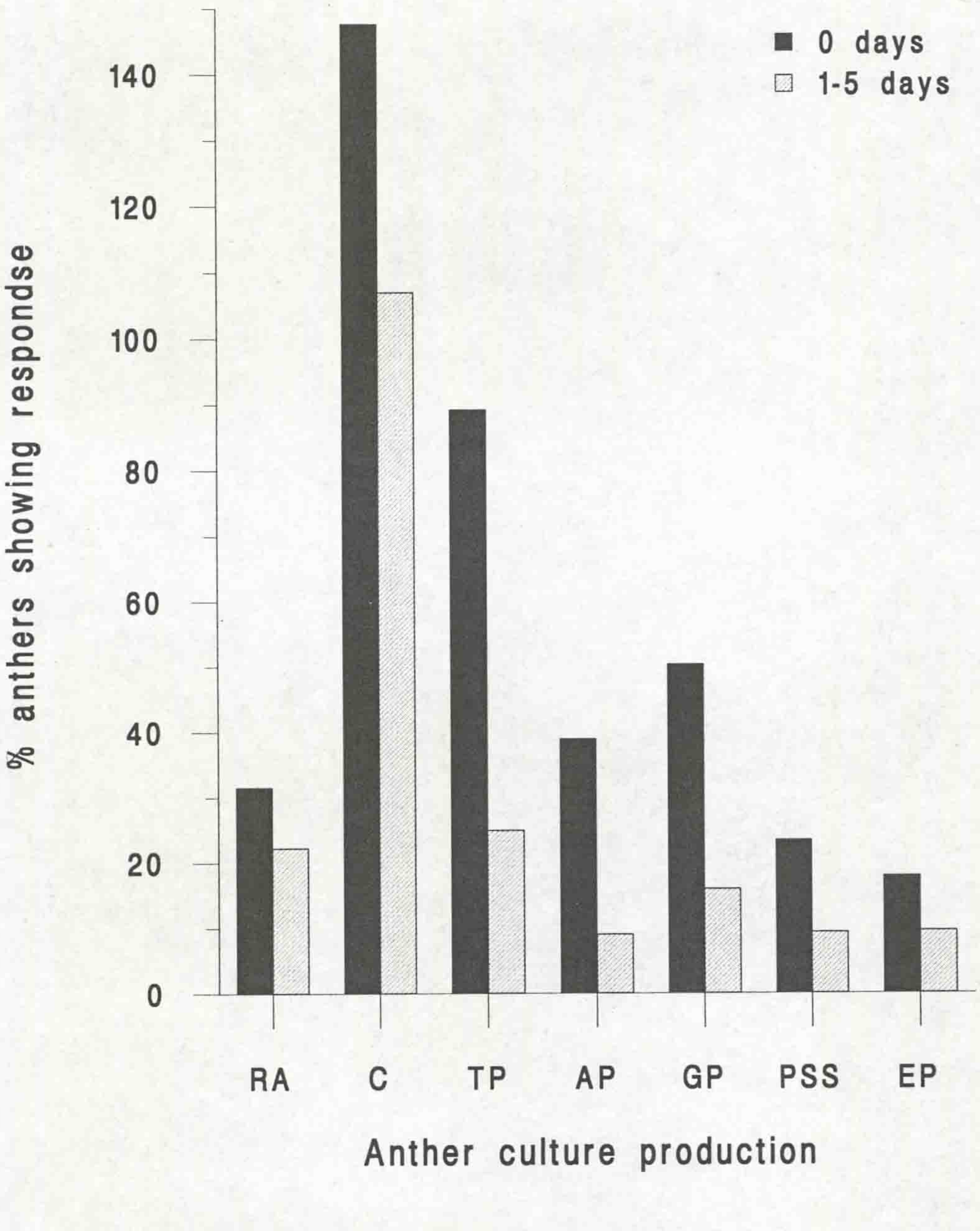


Figure 3.8. Effect of cold pre-treatment (days) on anther culture. RA = responding anthers, C = callus, TP = developed plantlets, AP = albino plantlets, GP = green plantlets, PSS = plantlets transferred to sterile soil and EP = established ACD plants.

The work carried out so far on anther culture of grasses, and interspecific and intergeneric hybrids of cereals and grasses has showed that production of regenerant plants (green and/or albino), and even callus production, is very dependent on genotype and medium. Only albino plantlets were regenerated from cultured *L. multiflorum* anthers at the uninucleate stage (Clapham, 1971) while Niizeki (1977) and Nitzsche and Wenzel (1977) obtained one green plant from anther culture of autotetraploid *L. multiflorum*. Anther culture response between diploid and tetraploid *L. multiflorum* was compared by Pagniez and Demarly (1979). They obtained albino plantlets and only one chimaerical white-green plant from tetraploid donor plants. Since no haploid plant and callus developed from the excised tall fescue anthers, green haploid plants were produced through anther-panicle culture of tall fescue (Kasperbauer *et al.*, 1980). Rose *et al.* (1987a) obtained only albino plantlets of *F. pratensis* by anther culture.

Halberg *et al.* (1990) identified 6 out of 55 hybrid clones from three responding diploid perennial ryegrasses with an anther culture response level superior to their parents. While the parents produced one green plant or less per 100 anthers, these superior clones produced from 11 to 59 green plants per 100 anthers.

Green plants from anthers were obtained in intergeneric grasses of *Lolium-Festuca* hybrids, *F. pratensis* × *L. multiflorum* (Nitzsche and Wenzel, 1977), *L. perenne* × *F. pratensis* and *L. multiflorum* × *F. pratensis* (Rose *et al.*, 1987a,b).

The main conclusion to be drawn from the present study is that microspores within cultured anthers of the *L. multiflorum* × *F. arundinacea* pentaploid hybrid (2n = 2x = 35) were capable of forming embryos from callus. Ward (1993) could not obtain green plants from triploid hybrids created by crossing the amphiploids Elmet (*L. multiflorum* × *F. pratensis* (4x) with *L. multiflorum* (2x) and Prior (*L. perenne* × *F. pratensis* (4x) with *L. perenne* (2x) with nearly the same medium used here. The amenability of the *Lm/Fa* pentaploid hybrid for androgenesis illustrated the potential for using anther culture of this hybrid genotype to select for novel gene combinations.

The physiological status of the plants at the time of anther excision strongly influenced the sporophytic potential of microspores in the anthers. The response in

culture is predominantly influenced by the different growth conditions. No morphological or developmental variation by environmental factors in the donor plant had any effect on the microspores as seen under the microscope. Cereal and grasses including *L. perenne* when grown in the field were more responsive than greenhouse grown material (Wenzel and Forowghi-Wehr, 1984) and perennial ryegrass (Olesen *et al.* 1988). Creemers-Molenar *et al.* (1988) also demonstrated that there was no consistent effect of growth conditions (field or glasshouse) of the donor plants on anther culture response in annual and perennial ryegrass.

The different artificial environmental and fertilizer combinations (see Table 3.1) on growth condition of the *Lm/Fa* pentaploid hybrid had some effect on all aspects of anther culture production. Height of the tiller of the panicle had no effect on anther culture production. However to gain the best results for anther culture, the donor plant should be grown in good soil in an unheated glasshouse and anthers should be chosen from the taller and more robust tillers. To extend the flowering time and thus increase the time for culturing anthers it was better to keep plants in a cool room with a short day length (8 hours) with no application of nitrogen fertilizer. Application of nitrogen fertilizer had a negative effect on anther culture productions.

Certain treatments given to whole spikes or to anthers can have a positive or negative effect on the development of the microspore. The most effective techniques used in anther culture are the use of a cold treatment on either cut inflorescences or excised anthers. Many researchers reported a positive effect of a cold pre-treatment on anther culture. Sunderland *et al.* (1981) recommend for barley a period of 21-25 days at 4°C or 14-21 days at 7°C for cut tillers. In rye a cold treatment of 6°C for a period of 3-15 days, depending on the plant genotype, was optimal (Wenzel *et al.*, 1977). In rice it has been observed that chilling or cold treatment or cold prior to culture enhances androgenesis (Zapata *et al.*, 1992). Sunderland and Roberts (1977) found that 12 days and longer at 7-8°C was optimal for buds of tobacco before excision. The reason for this positive androgenic effect following a cold pretreatment might arise because of an increase of microspores at first mitosis, as starch production is blocked. This increases

the opportunity for subsequent anther culture production (Wenzel and Foroughi-Wehr, 1984). Other researchers reported a negative effect of cold pre-treatment on anther culture. Hongchao *et al.*, (1988) considered cold pre-treatment (4°C) of whole spikes or separated anthers of wheat adversely affected the callusing efficiency of anthers. In the experiment described here, a cold pre-treatment on cut panicles of the *L. multiflorum* × *F. arundinacea* pentaploid hybrid for 1-5 days at 4 - 6°C also decreased all anther culture production.

The best pollen stage for anther culture of cereals and forage grasses is generally pollen grains at the mid to late uninucleate stage i.e. "e" to "f" stages of Figure 3.1 stated (Wenzel and Foroughi-Wehr, 1984; Olesen, 1987; Luckett and Smithard, 1992 and Heszky, 1992). Depending on species and even varieties, this stage can be before, during, or after the first mitosis of the microspore nucleus. The optimal developmental stage of microspores is early uninucleate in barley and mid uninucleate in wheat (Luckett and Darvey, 1992). In *Lm/Fa* pentaploid hybrid anthers at uninucleate and well vacuolated stage up to pre-mitotic stage (Figure 3.1, stages "e" to "i") were used which gave a good response to anther culture. Because of some variation in microspore development stage at time of culture, it was impossible to discriminate differences in androgenic success to precise stages of pollen microspore development.

However the *Lm/Fa* pentaploid hybrid was amenable to anther culture and 26.0% of anthers responded to culture. A total 2349 ACD plantlets developed from 3326 cultured anthers of which 57% were green and 43% albino. Percentage of green plantlets (40.3%) per 100 cultured anther in comparison with the production of green plantlets in F_1 hybrid of barley i.e. 10.4 (Finnie *et al.*, 1989), 1.39 (Luckett and Smithard, 1992), 1.8 to 7.6 per 100 anthers cultured (Kuhlmann and Foroughi-Wehr, 1989) was very promising. Over all 507 ACD plants (21.6% of cultured anthers) were established in the glasshouse. In this study the green : albino (1339:1010) was 133% (Table 3.7) while this ratio for barley was 27% (Finnie *et al.*, 1989) and 53% (Luckett and Smithard 1992).

CHAPTER FOUR

MORPHOLOGICAL DIVERSITY IN *L. MULTIFLORUM* × *F. ARUNDINACEA* PENTAPLOID HYBRID ANTHER CULTURE DERIVED PLANTS

4.1. Introduction

Visual evaluation of androgenic plants is useful to identify and eliminate obviously abnormal plants such as albinos. Also, there may be visually detectable superior characters such as leafiness, colour, and plant form (Kasperbauer, 1990b). This can begin soon after plant regeneration by eliminating albinos and those which develop obviously abnormal roots or shoots. Olesen (1987) considered the phase of establishment in the soil as the most sensitive stage in regeneration during anther culture. Androgenic plants showed a general slow growth, but displayed wide variation in vigour (Olesen, 1987). Ninety day old anther derived alfalfa plants grown in pots were evaluated for drought stress outside of glasshouse conditions for further 30 days (Safarnejad, 1996). All anther donor plants and most of the anther cultured derived plants died because of drought. The 6% which survived he considered were comparatively drought resistant plants. Theoretically, the use of cloned androgenic plants allows evaluation of the gametic clone under a wide range of field and other stress environments to identify those genetic combinations (haploid plants) that have the most desirable characteristics and to eliminate the undesirable ones (Kasperbauer, 1990b).

The wide range of chromosome combinations and chromosome recombinations generated by meiosis between *Lolium* and *Festuca* species might be expected to display in anther culture derived plants considerable diversity in morphology. Certain gene combinations may be entirely unique and offer potentially valuable agronomic characters suitable for commercial exploitation. Some of the morphological characters displayed in the androgenic population are described.

4.2. Materials and methods

Seven morphological characters were measured in the 88 anther culture derived plants and 12 control plants used in the field drought experiment (see section 2.3) just prior to drought treatment (i.e. under optimum growing conditions) in early June 1994. The seven morphological characters measured were:

(1) Height of the upper and tallest leaf from the soil surface (cm). This was used as an index of plant height.

(2) Tiller production index was assessed as the number of tillers produced over 75 days and expressed as a percentage.

(3) Variation in leaf roll morphology was categorised by visual scores on a scale of 1-5 (1 for completely rolled to 5 for leaf lamina completely unfolded).

(4) Leaf length (cm)

(5) Leaf width (mm).

Both leaf length (4) and leaf width (5) was based on means taken from the five most mature leaves of different tillers for each genotype.

(6) Flowering index.

In many temperate grasses there is an obligate winter requirement of several weeks of low temperature, vernalization, and short days for the induction of flowering (Jewiss, 1993). Thus all ACD plants were vernalizated (see Chapter 2). Since the youngest ACD plant (i.e. last ACD regenerant from culture), plant number 389, flowered extensively after 62 days vernalization, it was concluded that 62 or less days gave sufficient vernalization time for flowering for all ACD genotypes. Since vernalization for plant 389 ended on 1st. March 1994, the time between that date and first panicle emergence was taken as the index of flowering.

(7) Forage production expressed as dry matter yield.

Shoot biomass was harvested 6 cm approximately above ground level on 11th June 1994 and dried for 24 hours at 80°C. The herbage dry weight of each

individual genotype was recorded for each replicate and was used as a measure of forage production.

4.3. Analysis of data

Analysis of variance was used to assess the morphological diversity of ACD plants by use of four replicates in two field locations in which plants were grown, namely Field I was nominated for subsequent drought treatment and Field II would be used as the irrigated control (see Chapters 2 and 6 for details of the drought treatment). Analysis of variance (GLM type III) was calculated using SAS (Statistical Analysis System, 1990). Significant differences between treatments were compared by Ryan-Einot-Gabriel-Welsch multiple F-test and Boniferroni (Dunn) T-tests (Statistical Analysis System, 1990). Pairwise least significant differences (LSD) were calculated according to the Fisher method, which does not explicitly control experimental error, but was calculated to give a conservative estimate (Maxwell and Delaney, 1990). The formula was:

$$LSD = t_{edf,\ P <= 0.01} \times (2 \times EMS/n)^{0.5}$$

where: EMS = The error mean square from the analysis of variance

n = The number of replicates

t = The value of t-table for edf with P <= 0.01

edf = The error degrees of freedom

4.4. Results and discussion

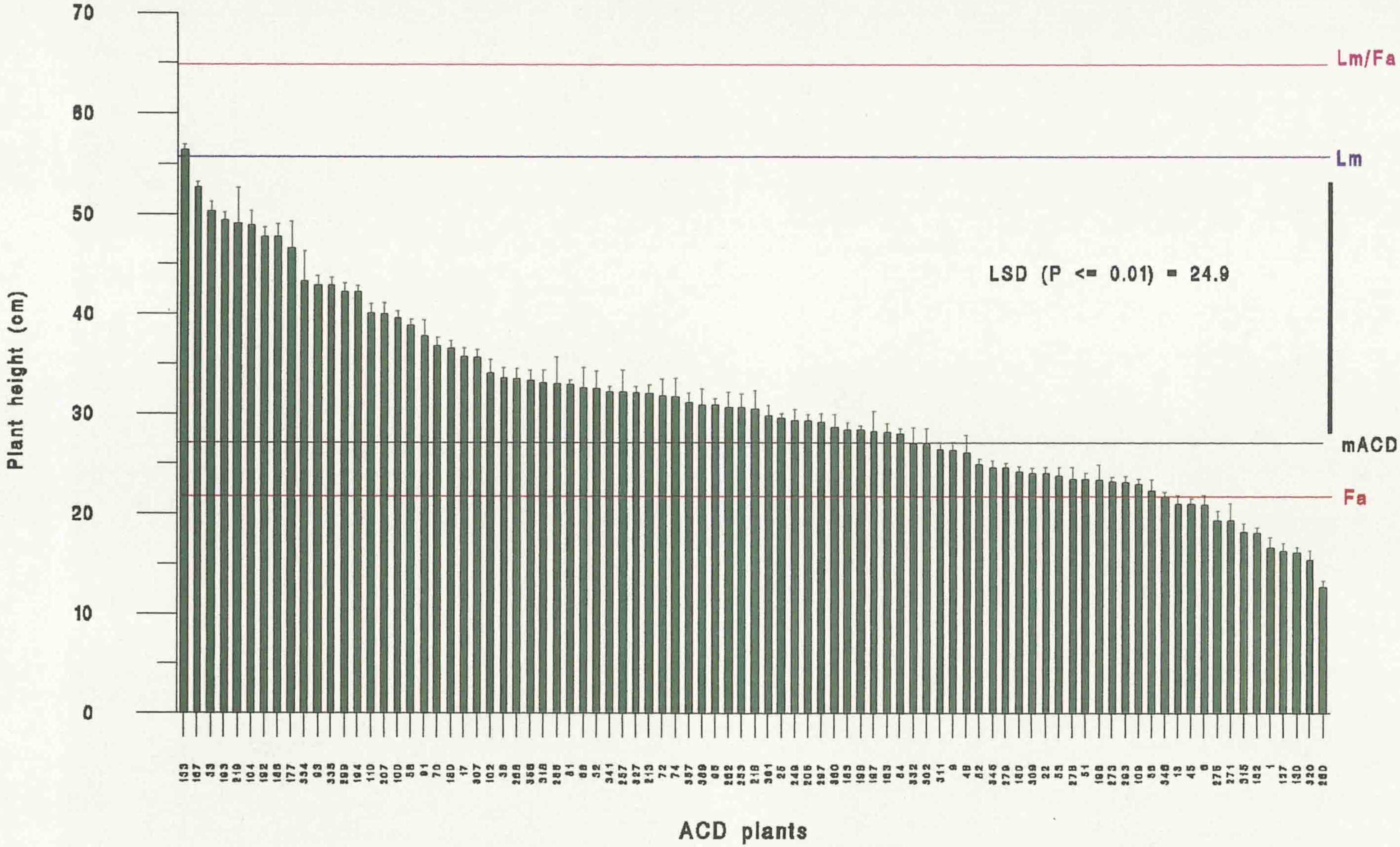
For centuries farmers have consciously chosen traits in their crops which appear to offer greatest potential for high yields. Plant breeding is simply an extension to this age old process e.g. morphological studies have been used as a tool for evaluation of wild tropical forage grass germplasm (Costa *et al.*, 1989). Yield

differed greatly among native populations, so that a preliminary choice of the outstanding ecotypes was made before starting the real breeding programme (Piano and Pusceddu, 1989). Breeding for improved forage quality is exceptionally difficult, and plant variability within tall fescue is insufficient for major improvements (Buckner *et al.*, 1967). Piano and Pusceddu (1982) characterized new varieties of tall fescue in spaced plants and clones by facultative summer dormancy, mid-season-late maturity, variable rhizome presence, long, relatively narrow and hanging leaves, rapid leaf elongation rate (particularly in winter), high number of ears per plant, good seed production, and relatively small seeds. This facilitated the breeding programme by reducing the number of crosses necessary to incorporate the desired characteristic because of small genetic distance between the progenitors. Interspecific and intergeneric crosses offered new sources of germplasm with increased variability from which superior varieties could originate (Buckner *et al.*, 1976).

Obvious variation in morphology was observed in the ACD plants in the field (Figure 4.1), including genotypes with characters that were absent in the parent species. The shoot and leaf colour of ACD plants ranged in appearance from bluish to light or dark green. Large variations between ACD plants were also found for the root biomass and the root systems. Differences in root morphology may have important implications for drought and cold tolerance. While worthy of a future major study, root dynamics in ACD plants and their relevance to environmental stress tolerance was not studied here.

Figure 4.1. Variation in general vigour of anther culture derived plants in the field.

Figure 4.2. Variation in plant height (cm) of ACD plants (Nos. 1-389). Lm = *L. multiflorum*, Fa = *F. arundinacea*, Lm/Fa = *L. multiflorum* × *F. arundinacea* pentaploid hybrid and mACD = mean performance of ACD plants.


70
60
50
40
30
20
10
0
Plant height (cm)
Lm/Fa
Lm
mACD
Fa
LSD (P <= 0.01) = 24.9
ACD plants

565

4.4.1. Plant height

Analysis of variance for ACD plant height (cm) at the beginning of flowering (after 88 days growth in the field) is presented in Appendix 4.1. ACD plants differed significantly ($P \leq 0.0001$) in plant height. The tallest ACD plant (No. 133) was 56.7 cm contrasting with the shortest plant (No. 260) which was only 7.2 cm in height. Overall the ACD plants had a mean height of 27.20 cm. The tallest ACD plant (No. 133) was taller (Figure 4.2) than both parental species but was shorter than the *Lm/Fa* pentaploid hybrid which is a particularly large and vigorous plant. However, the difference in plant height of Plant 133 was not statistically significant from the height of Italian ryegrass and the height of the *Lm/Fa* pentaploid hybrid. A total of 75 ACD plants were taller than tall fescue of which 9 of them (Nos. 133, 167, 33, 193, 219, 104, 192, 186 and 177) were significantly taller.

4.4.2. Tiller production capacity

Tiller density is an important growth component involved in production of forage grasses (Zarrough, 1983b). At low competition, and before equilibrium density of tillers was attained, forage production is determined largely by the number of tillers (Nelson *et al.*, 1977 and Zarrough *et al.*, 1983b). However, when tillering reaches equilibrium and density stabilizes, factors affecting forage production tend to be expressed mainly through yield /tiller (Langer, 1963; Nelson *et al.*, 1977 and Zarrough *et al.*, 1983b).

To evaluate variation among androgenic plants of the *Lm/Fa* pentaploid hybrid and their relationship to the parental species, an analysis of variance for ACD plants tiller production index was undertaken (Appendix 4.2). The ACD plants differed significantly ($P \leq 0.0001$) in tiller production capacity. The genotype with greatest tillering capacity, ACD plant 219 increased 7.35 fold in tiller

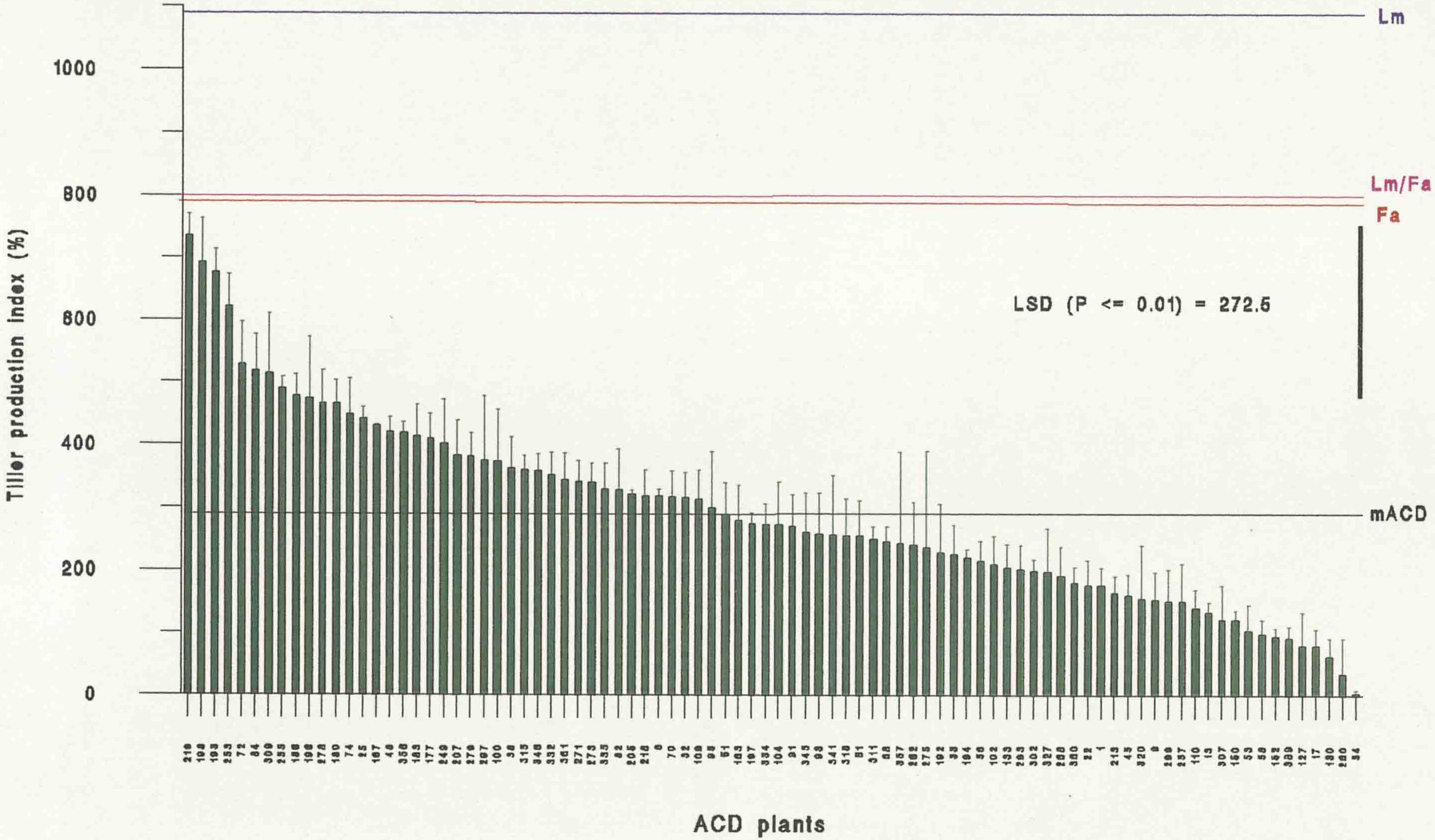
number over 75 days. On average all the ACD plants increased tiller number 3 fold. ACD plant No. 54 demonstrated little capacity to tiller. The tiller production of ACD plant 219 was equal to that of tall fescue and of the parental *Lm/Fa* pentaploid hybrid but was significantly smaller than Italian ryegrass (Figure 4.3).

4.4.3. Leaf shape

Analysis of variance for leaf shape in ACD plants at the start of flowering is presented in Appendix 4.3. Although ACD plants varied in leaf shape, 81 of the plants were similar to the controls in having fully opened leaf laminae. Figure 4.4 shows that the leaf shape of 5 ACD plants (Nos. 1, 127, 56, 256 and 198) which all demonstrated extensive amounts of leaf roll, differ significantly from the majority of ACD plants and the control genotypes.

Generally it can be concluded that the leaf shape in all ACD plants showed little diversity. This suggested that generally regenerants which demonstrated parental hybrid-like leaf morphology have a selective advantage in culture. De Buyser *et al.* (1985) observed meiotic abnormalities concerning chromosome numbers and/or structure in androgenic plants in wheat. These abnormalities were assumed to originate in the anther donor plants or during the process of anther culture. In 1300 androgenetic green plants of wheat, De Buyser *et al.* (1982) observed 10% hetroploid with loss or addition of chromosome or fragments. Aneuploid *Festulolium* plants frequently display leaf roll (M.W Humphreys, pers. comm.). Leaf roll in plants with chromosomal aberrations or aneuploid chromosome number (e.g. ACD No. 56 has 17 chromosomes) would therefore not be suppressing.

Figure 4.3. Variation in tiller production index (%) of ACD plants (Nos. 1-389). Lm = *L. multiflorum*, Fa = *F. arundinacea*, Lm/Fa = *L. multiflorum* × *F. arundinacea* pentaploid hybrid and mACD = mean performance of ACD plants.


Lm
Lm/Fa
Fa
LSD (P <= 0.01) = 272.5
mACD
1000
800
600
400
200
0
Tiller production index (%)
ACD plants

9/a

Figure 4.4. Variation in leaf shape (scores 1-5: 1 = rolled, 2 = nearly rolled, 3 = intermediate, 4 = partly expanded lamina and 5 = fully expanded lamina) of ACD plants (Nos. 1-389). Lm = *L. multiflorum*, Fa = *F. arundinacea*, Lm/Fa = *L. multiflorum* × *F. arundinacea* pentaploid hybrid and mACD = mean performance of ACD plants.

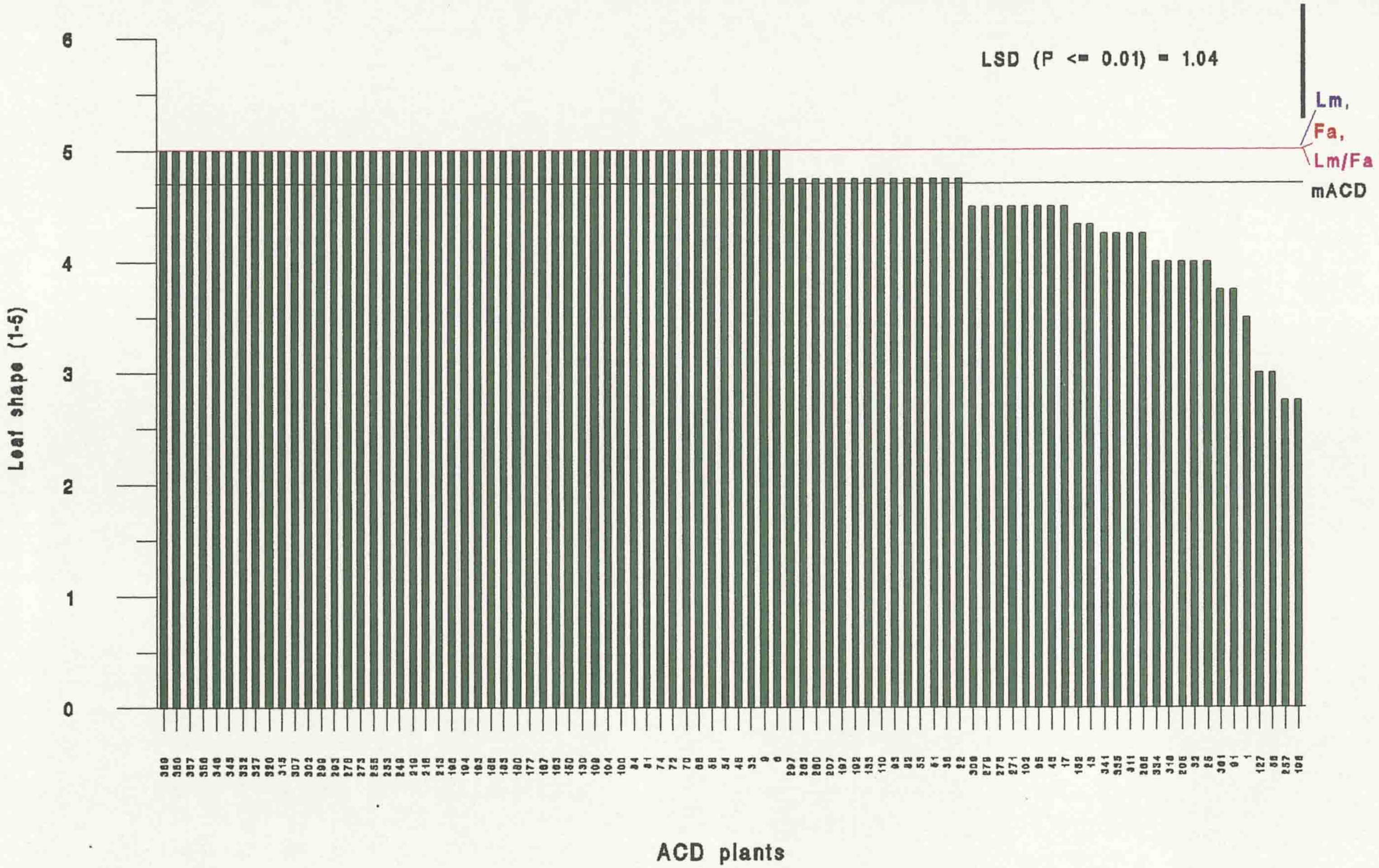
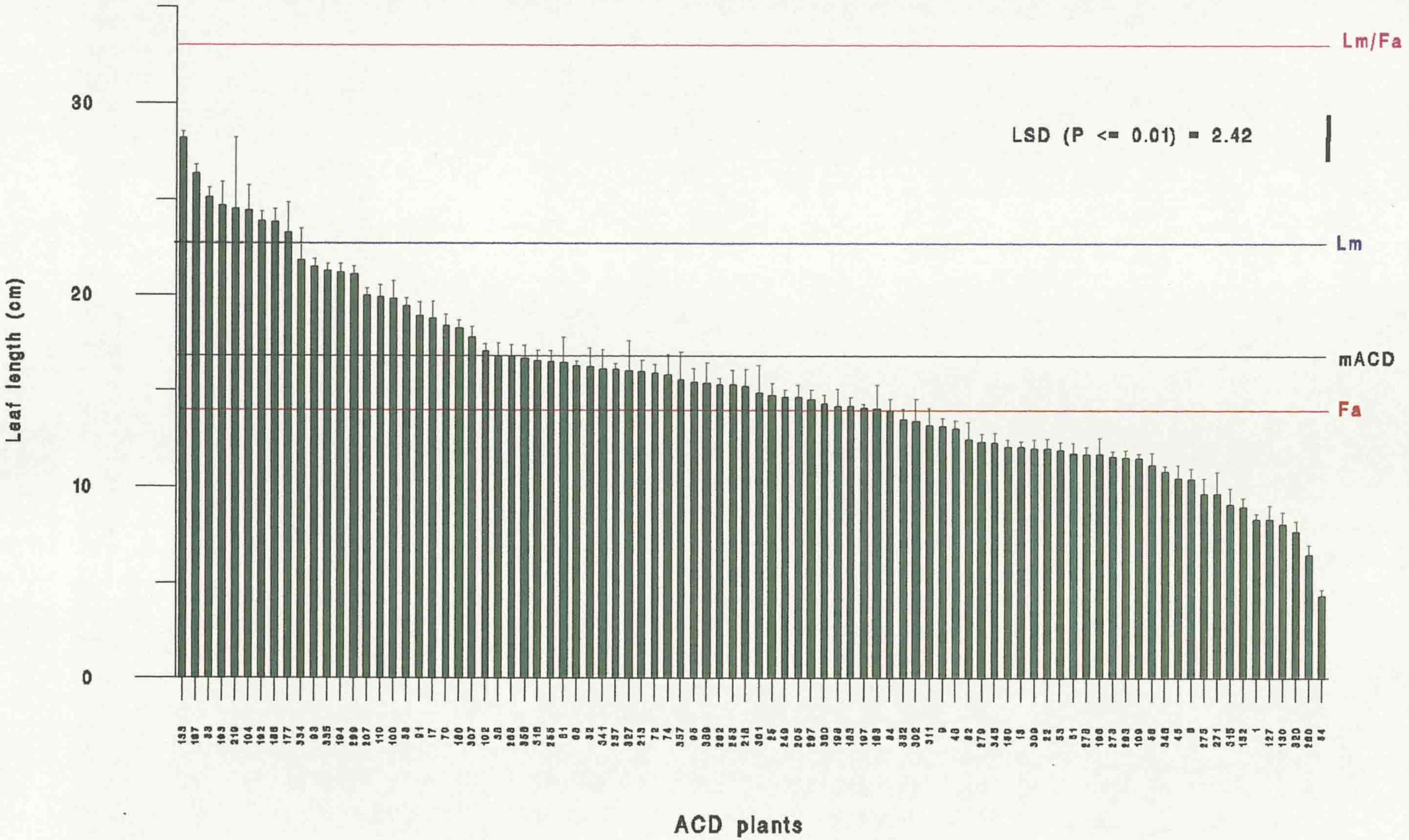

LSD (P <= 0.01) = 1.04
Lm,
Fa,
Lm/Fa
mACD
Leaf shape (1-5)
6
5
4
3
2
1
0
ACD plants

Figure 4.5. Variation in leaf length (cm) of ACD plants (Nos. 1-389). Lm = *L. multiflorum*, Fa = *F. arundinacea*, Lm/Fa = *L. multiflorum* × *F. arundinacea* pentaploid hybrid and mACD = mean performance of ACD plants.


Lm/Fa
LSD (P <= 0.01) = 2.42
Lm
mACD
Fa
30
20
10
0
Leaf length (cm)
ACD plants

4.4.4. Leaf length

Analysis of variance for leaf length is presented in Appendix 4.4. There was considerable variation between ACD plants in leaf length. The leaves of plants Nos. 133 and 167 were significantly longer than the leaves of both parental species. There were a further 21 ACD plants with leaves longer than *F. arundinacea*. The mean leaf length overall was significantly shorter than that of *L. multiflorum* but longer than that of *F. arundinacea* (Figure 4.5). The leaves of the ACD plant 133 were more than 3 times longer than the leaves of plant 54 (with shortest leaves). The *Lm/Fa* pentaploid had significantly longer leaves than all other ACD plants and control genotypes.

4.4.5. Leaf width

Analysis of variance in leaf width of ACD plants indicated significant differences between genotypes Appendix 4.5. Although the mean leaf width of the ACD plants was narrower than *L. multiflorum,* it was not significantly narrower than *F. arundinacea* but the leaves of *Lm/Fa* pentaploid were significantly broader than both species and ACD plants except for ACD plant Nos. 104 and 299 (Figure 4.6). Plants, 104, 299, 110, 327, 194, 167 and 177 had broader leaves than either parental species. Conversely, 20 other ACD plants e.g. Nos. 100, 72, 268 and 102 had broader leaves than *F. arundinacea*.

4.4.6. Flowering

Since flowering is a shifting of the vegetative phase to a reproductive phase Wilkins (1994) suggested that flowering intensity may be the primary factor controlling seasonal yield distribution of perennial ryegrass varieties and their suitability for different production systems.

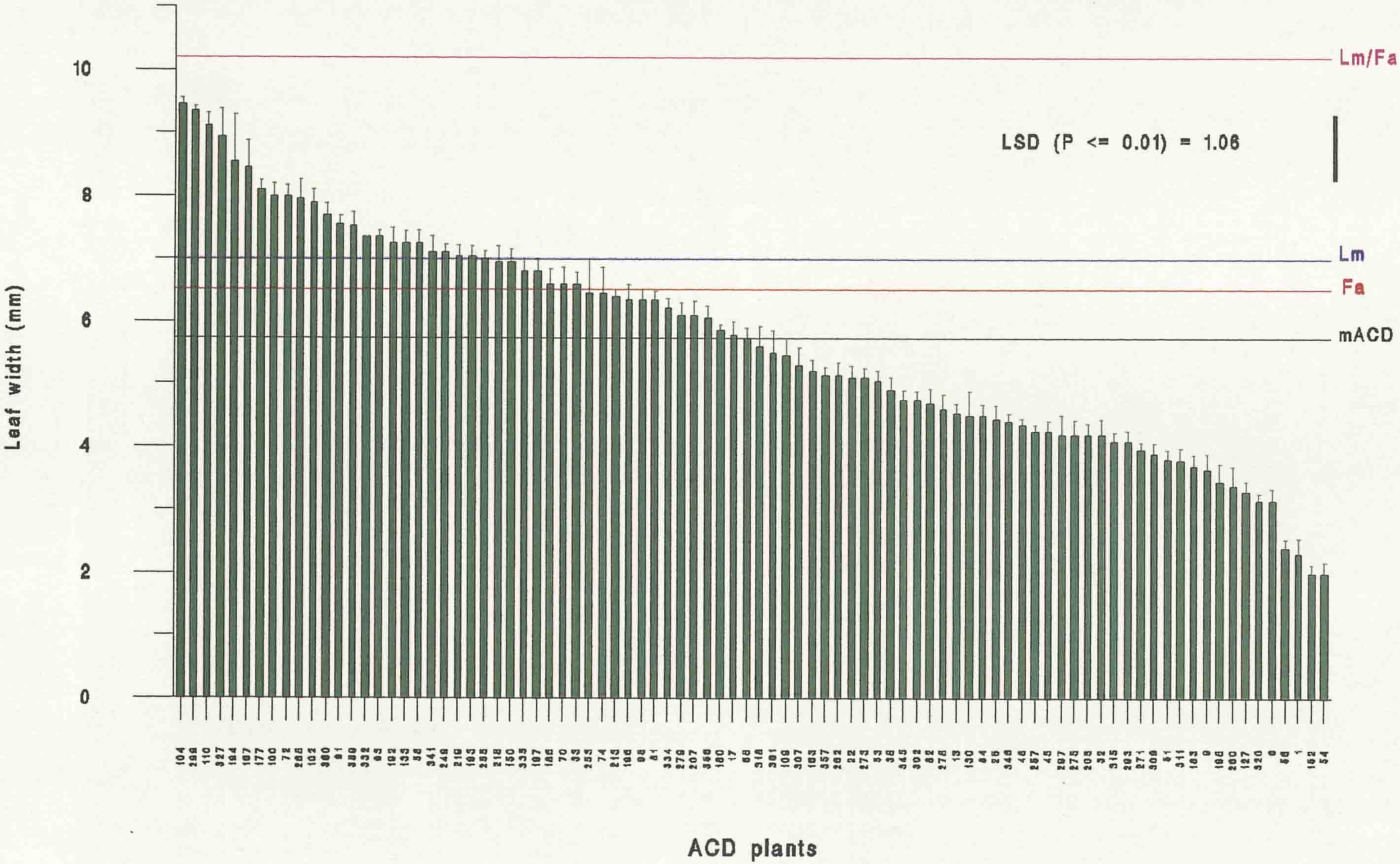
In spite of sufficient vernalization to enable flowering, under half of the ACD plants (39 plants) flowered. For future exploitation, it will be essential to achieve flowering of any selected androgenic genotype to produce progeny for commercial use. Analysis of variance of the flowering index is presented in Appendix 4.6. ACD plants differed greatly ($P \leq 0.0001$) in flowering index. No ACD plant flowered earlier than Italian ryegrass and *Lm/Fa* pentaploid hybrid (Figure 4.7). Only one plant of tall fescue flowered at Cut 1 (11th June), which was later than both Italian ryegrass and the *Lm/Fa* pentaploid hybrid. Flowering index of the *Lm/Fa* pentaploid hybrid was not statistically later than Italian ryegrass but it flowered earlier than tall fescue. Since some of the tall fescue plants flowered at Cut 2, probably if ACD plants could not be cut, some more ACD plant might be flower. However among the ACD plants which flowered, there was 30 days difference between the earliest flowering ACD plant (102) and the latest flowered ACD plant No. 56. The reasons for late flowering or no flowering in the case of most androgenic plants was not clear. Some of the polyhaploid plants (e.g. No. 219) did not flower after 3 years and may require additional treatments e.g. with Gibberellic acid (GA3) (Jones, pers. comm.) to initiate flowering response.

4.4.7. Herbage yield

Development of effective selection criteria for the improvement of dry matter is desirable in forage grass breeding (Reeder *et al.*, 1984, Zarrough *et al.*, 1983a; Zarrough *et al.*, 1983b). Genotypes in the vegetative condition differed in their means of achieving yield (Nelson *et al.*, 1977). Dry mater production of tall fescue is usually analysed for CO_2 metabolism, leaf area index, and morphological traits (Gáborcik, 1989). Genotypes selected for low, medium, and high yield/tiller in spaced plant studies exhibited a high, medium and low rate of tillering, respectively ((Zarrough, 1983a&d).

Analysis of variance of the dry herbage yield (g) of ACD plants is presented in Appendix 4.7. ACD plants differed significantly in herbage yield. ACD plant No. 193 (the most high yielding) produced 22.7 g herbage dry matter while No. 54 produced only 0.03 g herbage dry matter. The *Lm*/*Fa* pentaploid hybrid produced significantly more dry herbage yield than all of the ACD plants, ryegrass and tall fescue. The *L. multiflorum* produced significantly more dry herbage yield than all of the ACD plants and the *F. arundinacea* (Figure 4.8). There were 10 ACD plants which produced more herbage yield than the *F. arundinacea* parent, which in the case of 7 plants (Nos. 193, 167, 219, 327, 360, 186, 133 and 389) was significant.

Figure 4.6. Variation in leaf width (mm) of ACD plants (Nos. 1-389). Lm = *L. multiflorum*, Fa = *F. arundinacea*, Lm/Fa = *L. multiflorum* × *F. arundinacea* pentaploid hybrid and mACD = mean performance of ACD plants.



97a

Figure 4.7. Variation in flowering index (days) of ACD plants (Nos. 1-389). Lm = *L. multiflorum*, Fa = *F. arundinacea*, Lm/Fa = *L. multiflorum* x *F. arundinacea* pentaploid hybrid and mACD = mean performance of ACD plants.

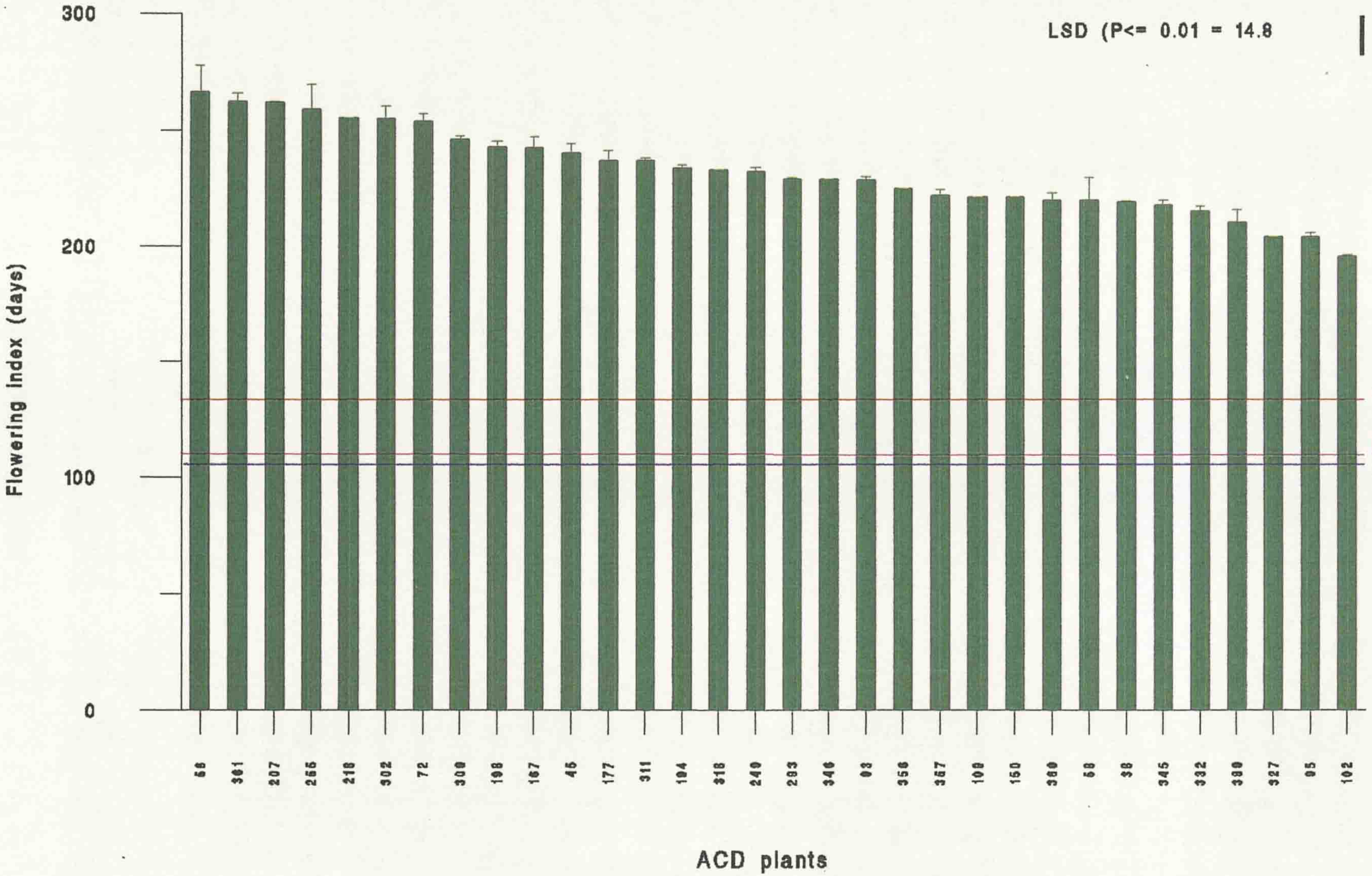
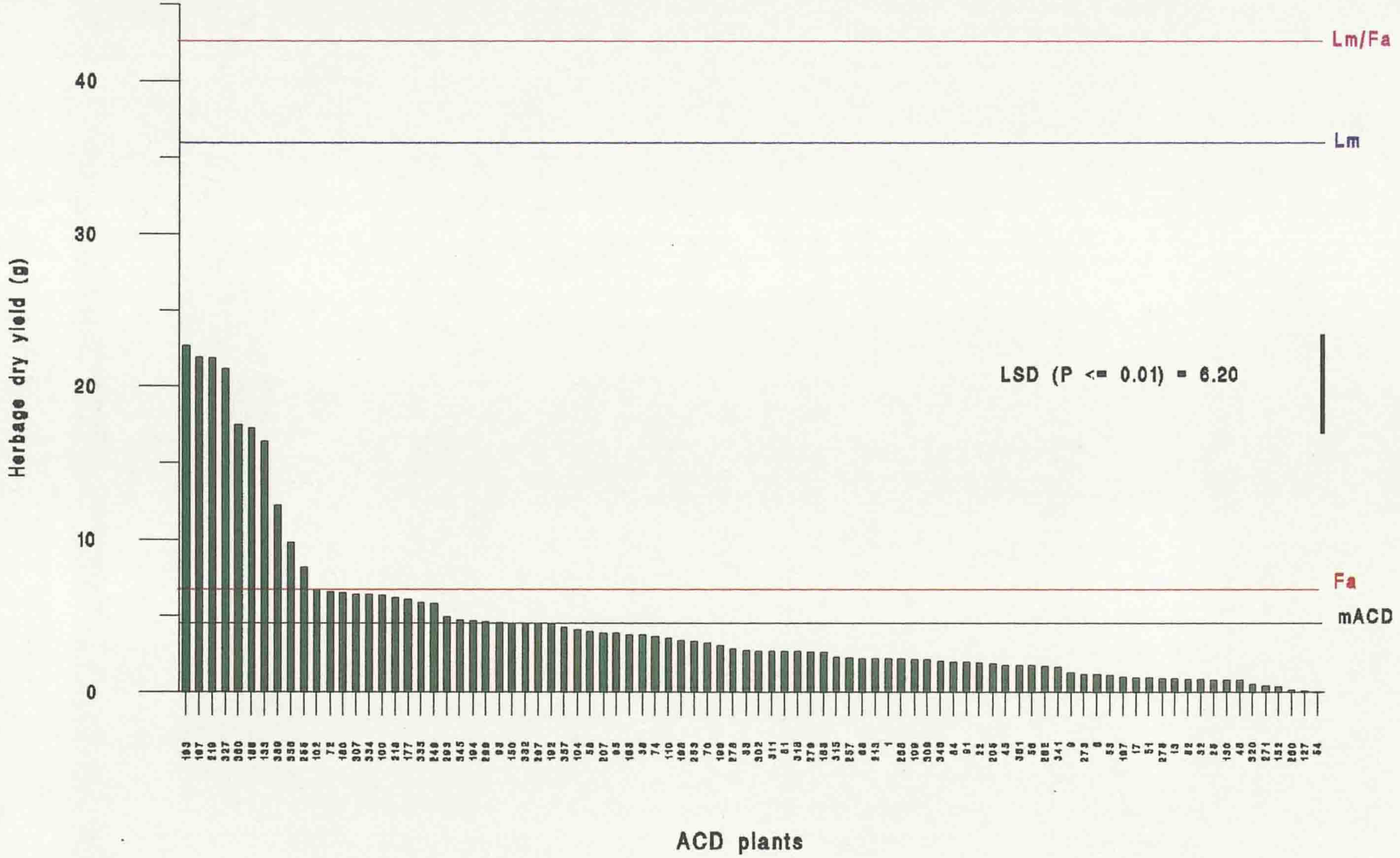

LSD (P<= 0.01 = 14.8
300
200
100
0
Flowering Index (days)
Fa
Lm/Fa
Lm
56
361
207
265
218
302
72
300
198
167
45
177
311
194
318
240
283
346
63
356
367
100
150
380
58
38
345
332
380
327
95
102
ACD plants

Figure 4.8. Variation in herbage dry matter (g) of ACD plants (Nos. 1-389). Lm = *L. multiflorum*, Fa = *F. arundinacea*, Lm/Fa = *L. multiflorum* × *F. arundinacea* pentaploid hybrid and mACD = mean performance of ACD plants.


Lm/Fa
Lm
40
30
20
10
0
Herbage dry yield (g)
LSD (P <= 0.01) = 6.20
Fa
mACD
ACD plants

4.4.8. Correlation amongst selected morphological characters

It was anticipated that anther culture might reveal variation in plant morphology unavailable within *L. multiflorum* and *F. arundinacea* species which might have agronomic value and could be exploited later commercially. Development of effective selection criteria for the improvement of dry matter is a goal in forage grass breeding (Reeder *et al.*, 1984). Selection for one morphological character might have a correlation (positive or negative) with the other morphological characters. Ceccarelli *et al.* (1980) in perennial ryegrass, found for example selection for high dry matter did not change leaf size, but was associated with increased rate of tiller production with no change in rate of leaf appearance, or selection for low dry matter yield and reduced rate of tiller production. The seven morphological characters (plant height, tiller production capacity, leaf dimension and shape, flowering habit and herbage dry matter yield) of ACD plants were examined for possible correlation.

4.4.8 1. Pearson correlation coefficients

Pearson correlation coefficients for all six morphological characters are shown in Table 4.1a. This indicated various positive correlations between morphological traits. Three groups can be separated in which there are little, moderate and strong positive correlations amongst the characters for a particular group:

Group I. Dry matter yield, plant height, leaf length and leaf width have a strong positive correlations amongst themselves ($0.52118 \leq R \leq 0.73216$).

Group II. There are moderate positive correlations between tiller production capacity with dry matter yield and leaf length and also the correlations of leaf shape with leaf width and dry matter yield ($0.26408 \leq R \leq 0.35171$).

Group III. There were no statistically significant correlations between plant height and tiller production capacity and leaf shape, between tiller production

capacity with leaf width and leaf shape and also between leaf length and leaf shape ($0.08298 \leq R \leq 0.18747$).

4.4.8.2. Principal component analysis[1]

Principal component (SAS 1990) analysis was used to analyse plant phenotypes. Results are presented in Tables 4.1b&c. The first component is a measure of overall size variation, since the first eigenvector[2] (PRIN 1) shows approximately equal loading (0.453645 - 0.492220) on the four characters dry yield, plant height, leaf length and leaf width. This component therefore seems to reflect levels of natural plant vigour. The second eigenvector (PRIN 2) has very high positive loading on tiller production capacity and the third eigenvector (PRIN 3) has very high positive loading on leaf shape. The interpretation of remaining eigenvectors is not obvious (Table 4.1c).

Sixty-eight percent of the variation present in the principal component analysis is explained by the first two eigenvectors (Table 4.1b). The first eigenvector accounting for 51.2% of variation (Table 4.1b) would appear to be positively correlated with dry matter yield, plant height, leaf length and leaf width and had a moderate association with leaf shape and tiller production capacity. The second eigenvector accounting for 16.7% (Table 4.1b) of the variation was strongly associated with variation in tiller production capacity and was moderately negatively associated with plant height, leaf width and leaf shape. The third eigenvector accounts for 15.5% (Table 4.1b) of the total variation and had the highest loading on the leaf shape component.

Since the leaf shape showed restricted variation (Figure 4.4) only the first two eigenvectors (PRIN 1 and PRIN 2) were used to summarize the data. It is possible to

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[1]Principal component analysis is a multivariate technique for examining relationships among several quantitative variables. Principal components have been used to reduce the 6 morphological variables (plant height, tiller production index, leaf shape, leaf length, leaf width and herbage dry matter) in to 2 components (PRIN1 and PRIN2).

[2]The eigenvalues and eigenvectors of matrices form an axis used to achieve dimension reduction by defining new variables called principal components.

identify a trend amongst the ACD plants from small low yielding plants e.g. Nos. 54 and 260 to large and very robust plants e.g. Nos. 219, 193, 167 and 186 (Figure 4.10). Leaf area shape rate may be an acceptable criterion for yield/tiller (Nelson *et al.*, 1977). Reeder *et al.* (1984) reported gene action for leaf area shape rate and its components are largely additive. The best ACD plants from an agronomic point of view e.g. Nos. 219 and 193 were those plants with large dry matter yield, resulting from a tall height, long and wide leaves and also with high tiller production capacity. In tall fescue tiller/plant was negatively correlated (r = -0.55) with yield/tiller (Nelson *et al.*, 1977). They found rate of leaf area shape was related closely to yield/tiller among genotypes that were selected to represent a broad range in number of tillers/plant.

However, overall the results of morphological characters of androgenic plants were in agreement with Riesesberg *et al.*, (1993). He concluded that from molecular and morphological markers of plant hybridization, expression in the hybrid represented a mosaic of both parental and intermediate characters rather than strictly intermediate ones.

It can be concluded that by means of anther culture large variation in plant morphology was demonstrated in pollen regenerants from *L. multiflorum* × *F. arundinacea* pentaploid hybrid.

Table 4.1: Principal component analysis of six morphological ACD plants characters

a) Correlation matrix

Pearson correlation coefficients and probability of Ho in parenthesis; DY = dry yield, PH = plant height, TPC = tiller production capacity, LL = leaf length, LW = leaf width and LS = leaf shape.

	DY	PH	TPC	LL	LW	LS
PH	0.57641	1.0000				
	(0.0001)					
TPC	0.33486	0.08298	1.0000			
	(0.0014)	(0.4421)				
LL	0.58491	0.65593	0.28080	1.0000		
	(0.0001)	(0.0001)	(0.0080)			
LW	0.52118	0.67408	0.12860	0.73216	1.0000	
	(0.0001)	(0.0001)	(0.2324)	(0.0001)		
LS	0.26408	0.18747	0.08469	0.14098	0.35171	1.0000
	(0.0129)	0.0803)	(0.4344)	(0.1901)	(0.0008)	

b) Eigenvalues of the correlation matrix

	Eigenvalue	Difference	Proportion	Cumulative
PRIN 1	3.07328	2.07117	0.512214	0.51221
PRIN 2	1.00211	0.07537	0.167018	0.67923
PRIN 3	0.92674	0.45692	0.154457	0.83369
PRIN 4	0.46982	0.15550	0.078303	0.91199
PRIN 5	0.31432	0.10060	0.052387	0.96438
PRIN 6	0.21372	-	0.035620	1.00000

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c) Eigenvectors

	PRIN 1	PRIN 2	PRIN 3	PRIN 4	PRIN 5	PRIN 6
DY	0.453645	0.185688	0.016615	-0.785914	-0.348770	-0.141944
PH	0.469810	-0.238977	-0.237665	-0.161724	0.792063	0.110295
TPC	0.198362	0.899283	0.147964	0.232446	0.258777	-0.095149
LL	0.492220	0.026976	-0.261731	0.337557	-0.384401	0.653284
LW	0.491100	-0.239868	-0.008730	0.432413	-0.181388	-0.693776
LS	0.225117	-0.203559	0.923454	0.035034	0.057997	0.224785

PRIN = Principal component analysis, DY = Dried yield, PH = Plant height, TPC = Tiller production capacity, LL = Leaf length, LW = Leaf width and LS = Leaf shape.

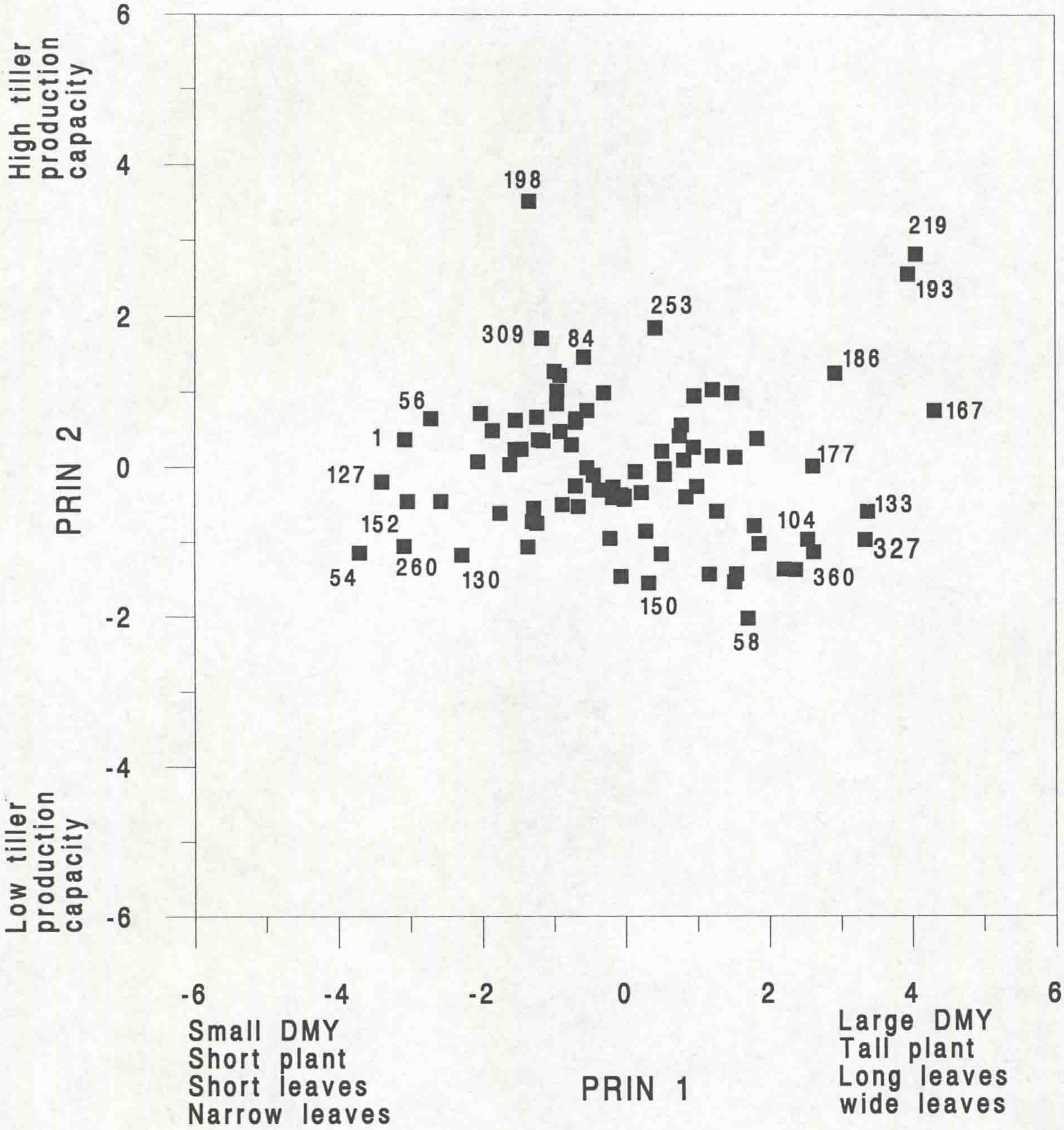


Figure 4.9. Plot of principal component 1 (herbage dry yield, plant height, leaf length and leaf width) × principal component 2 (tiller production index) of ACD plants. Selected ACD plants (Nos. 1-389) are labelled.

CHAPTER FIVE

THE EFFECT OF FREEZING STRESS ON *L. MULTIFLORUM* × *F. ARUNDINACEA* PENTAPLOID ANTHER CULTURE DERIVED PLANTS

5.1. Introduction

Low temperatures are a limiting factor for much of plant growth and is a determinant of its distribution. Low temperatures decreases the biosynthetic activity of plants and the normal function of physiological processes and may even initiate permanent injuries, that finally bring about death (Alberdi and Corcuera, 1991).

Plant species have been classified into three groups based on their ability to withstand cold. They are: a) Chilling-sensitive plants that suffer injury from temperature decrease, usually to 10-15°C, b) freezing-sensitive plants that suffer injury at temperatures below 0°C, and c) freezing-resistant plants that are able to survive subzero temperature down to a limit which is characteristic of the genotype (Larcher, 1981). Super freezing tolerance can be seen in some fully hardened woody plants where their cells can tolerate exposure to temperature of -50 to -100°C (Taiz and Zeiger, 1991).

Winter hardiness in cereals is the consequence of a number of complex and interacting component characters: cold tolerance, vernalization requirement, and photoperiod sensitivity (Pan *et al.*, 1994). More effective selection should be possible by understanding the genetic basis of these component traits. Almost all reports emphasise that cold tolerance is a quantitative character in which genes on many chromosomes are involved (e.g. Sutka, 1981; Norell *et al.*, 1986; Sutka, Veisz and Kovacs, 1986; Limin and Fowler, 1988 and Larsen 1994). In wheat and barley, several studies have reported evidence that winter hardiness is controlled by partially dominant and/or recessive genes (Rohde and Pulham, 1960; Law and Jenkins, 1970 and Sutka 1981), which appear to act under different levels of stress in an additive fashion (Rohde and Pulham, 1960 and Gullord, Olien and Everson, 1975). Also cytoplasmic cold tolerance inheritance in rice has been reported (Ratho and Pradhan, 1992). Between temperate grain cereals, the ranking in decreasing order of cold tolerance is rye > wheat > barley > oats (Blum, 1988).

Considerable variation in cold tolerance can exists among ecotypes of a single species (Lorenzetti *et al.*, 1971; Fuller and Eagles, 1978 and 1980; Charles *et al.*, 1981; Jones and Charles, 1984; Blum, 1988; Pollock and Eagles, 1988; Eagles, 1989; Taiz and Zeiger, 1991 and Salisbury and Ross, 1992). Some species are able to survive harsh environments, while closely related species are sensitive to frost and cold (Alberdi and Corcuera, 1991). An example of the latter group is the *Lolium/Festuca* complex where *Festuca* species are generally more cold resistant than those of *Lolium*. Improvement of winter survival is a high priority in perennial grass breeding programmes for northern temperate climates (Breese and Foster, 1970; Hides, 1979; Larsen, 1985 and Humphreys and Eagles 1988). Many grasslands are hill-lands (approximately 4.76 million acres in the U.K.) classified as "rough hill grazing" (Woodford and Morrison, 1971). As a result of increasing social and economic pressure on good lowland soils both for cereal crop farming and from development of urban and recreational sites, hill-land has become increasingly important as a source of fodder for animal production. However, hill-land productivity is appreciably lower than in lowland areas because it offers a shorter growing season, lower temperatures and generally poorer soils and possible waterlogging. Also species best adapted to hill-lands are frequently inferior to lowland species as a source of fodder for the grazing animal. In theory, the quality of hill-land pastures may be increased by replacing the existing ecotypes with agronomically more desirable species (Davies, Edwards and Rowlands, 1968 and Newbould 1974). However, low winter temperatures normally limit the use of agronomically valuable grass species for use in upland improvement which inherently are less winter hardy than species native to the ecosystem. Varieties of good quality *Lolium* species with increased winter hardiness would be valuable in any hill-land improvement scheme where the aim is for stable pastures accompanied by high productivity.

In practical terms, minor increases in winter-hardiness could have a major impact on world food production. Winter wheats and rye yield 25 to 40 percent more

than comparable spring cultivars because they make better use of spring rains. If the winter wheats and rye could be made 2°C more cold hardy, they could replace much of the large areas of spring wheats and rye in North America and Russia (Salisbury and Ross, 1992). Developing forage grasses and legume cultivars with potential growth in the autumn and spring is one of the breeding objective in the North Europe (Eagles and Fuller, 1982) and also in other temperate climates. This out of season growth may be achieved by exploiting the winter hardy characteristics of winter hardy populations of the same or relevant species by hybridization with native adapted varieties. By hybridisation with northern-adapted types and subsequent selection, this out of season growth has been obtained in hybrid varieties such as cv. Saborto (*Dactylis glomerata* hybrid between S.37 and a Portuguese diploid) and cv. Katrina (*Trifolium repens* hybrid between Kersey, Israeli and Turkish types) (Eagles and Fuller, 1982).

Grassland improvement has led to the establishment of pastures dominated primarily by high yielding digestible varieties of ryegrass. Italian ryegrass varieties offer the most nutritious forage but unfortunately have an inherent problem of lack of winter hardiness. Tall fescue however, is comparatively cold tolerant compared with ryegrass (Thomas and Humphreys, 1991). It is likely that within the androgenic population developed from the *L. multiflorum* × *F. arundinacea* pentaploid hybrid there exists a range of freezing tolerance from the low temperature tolerance of the *Festuca* parent to the more freezing sensitive *Lolium*. The aim of the work described herein was to assess the range of cold resistance within the androgenic plant population and to assess the potential of anther culture as a technique for selecting gene combinations which confer particularly high tolerance to freezing stress.

5.2. Materials and methods

5.2.1. Plant materials

Tillers of 47 androgenic plants and of their parents as controls i.e. *L. multiflorum*, *F. arundinacea* and the *L. multiflorum* × *F. arundinacea* pentaploid hybrid, were used to assess freezing tolerance.

All plants were cold acclimated at a constant temperature of +2°C an 8-h day and a photosynthetic photon flux density (PPFD) of 300 - 315 μmol m^{-2} s^{-1} for 2 weeks.

5.2.2. Measurement of freezing tolerance

Freezing tolerance was measured using a freezing tank (Fuller and Eagles, 1978) consisting of an insulated steel bath (155 cm × 118 cm × 50 cm) containing 50% (v/v) polyethylene glycol (PEG) and water. This PEG-water mixture was cooled by refrigeration and circulated by a pump. Individual tillers of cold-acclimated plants were separated from their mother plant and loaded singly into sets of 5 perspex tubes 20.5 cm long × 2.3 cm diameter isolated from the PEG-water mixture. A set of perspex tubes, each containing 5 tillers of 1 genotype was used for 5 freezing temperature treatments (total of 25 tillers per genotype). Each set of perspex tubes with tillers was placed into the freezing tank prior to freezing at +2°C. Overnight the temperature in the freezing tank was gradually reduced to the initial freezing temperature treatment of -2°C. A thick sheet of polystyrene, about 15 cm deep was placed over the freezing tank for insulation. The freezing temperatures used to calculate LT_{50} were taken at 3°C intervals between -2 °C and -14°C. A set of perspex tubes, i.e. 5 tillers per genotype was removed from the freezing tank on reaching each designated temperature treatment (-2°C, -5°C, -8°C, -11°C, and -14°C).

Plant response to freezing stress was known to remain unchanged if genotypes were assessed on different days (Eagles pers. comm.). In order to accommodate the

numbers of ACD plants to be tested, the experiment was split up over two days. The genotypes were split into groups of 24 ACD plants plus control genotypes in the first freezing test and the 23 ACD plants plus control genotypes in the second experiment.

Following freezing treatment, tillers were planted in shallow multitrays containing John Innes No. 3 compost. Tiller survival was scored after a three week recovery period in a growth room maintained at +15°C and 8-h day length and with regular daily watering.

5.3. Results and discussion

Researchers at IGER (Institute of Grassland and Environmental Research), found a close relationships between winter hardiness under field conditions and cold tolerance of seedlings or mature plants of grasses, white clover and winter oats (Fuller, 1979 and Eagles and Fuller, 1982) measured by environmental simulation using a glycol freezing tank and expressed as the lethal temperature for 50% kill (LT_{50}). Freezing tolerance has been identified as a potentially useful component of winter hardiness in breeding programmes, provided that appropriate hardening conditions are imposed (Cooper, 1964; Breese and Foster, 1970; Lorenzetti *et al.*, 1971 and Hides, 1979). With all assessments of cold tolerance, the ultimate criterion must be the ability to survive under natural conditions. However, the irregularity and unpredictability of severe winters in many regions such as western Britain limits the use of field testing and cannot be considered ideal for any assessment of potential breeding material. In the absence of reliable natural freezing conditions an isolated tiller test for cold tolerance based on a technique used for winter cereals (Jenkins and Roffey, 1974 and Fuller and Eagles, 1978) is now an accepted test procedure and is commonly used for cold tolerance assessment in forage grasses. Tests for freezing tolerance are considered to give the best indication of overall winter survival for Gramineous plants and can be used as with any other quantitative characters in a breeding programme (Larsen, 1994).

Fig. 5.1. Surviving tillers of ACD plants after three weeks recovery following exposure to -8°C (a) and -14°C (b) temperature treatments.

With very few exceptions tillers of all androgenic genotypes recovered following freezing at -2°C. One exception was plant 293 which had only 60% tiller survival at this temperature. The consistent recovery throughout the experiment irrespective of genotype at -2°C implies that any death of tillers at lower temperature treatments was due to their susceptibility to freezing rather than from accidental damage resulting from the procedures employed.

Tiller death resulting from freezing damage increased at -5°C indicating some susceptibility to freezing to this temperature. Tiller death was further increased at -8° C (Figure 5.1a) and only a few androgenic genotypes survived at -11°C. With the exception of three genotypes, all tillers died at -14°C (Figure 5.1b). Only ACD plant 219 had 100% recovery following all freezing treatments.

Survival following each temperature treatment was determined on the basis of lethal temperature to 50% tillers (LT_{50}) for each genotype. LT_{50} values (Table 5.1) were calculated by two procedures:

a) probit analysis (Maximum Likelihood Program, Ross 1987), where sufficient intermediate responses to freezing were found within genotypes (found in 15 ACD plants and all three control genotypes). Analysis involved plotting the probit of the mortality response of a genotype against the logarithm of temperature survival. Regression analysis was then used to estimate the LT_{50} (Maximum Likelihood Program, Ross 1987).

b) graphical means.

Table 5.1. The lethal temperature of 50% kill (LT_{50}) values. (P = LT_{50} calculated by probit analysis; G = LT_{50} determined by graphical means.)

Genotypes	P	G	Genotypes	P	G
ACD 1	-7.17	-7.2	ACD 255	-	-8.8
ACD 6	-	-4.5	ACD 257	-	-6.9
ACD 9	-	-5.2	ACD 275	-	-5.5
ACD 45	-	-6.5	ACD 278	-6.99	-9.1
ACD 56	-	-6.6	ACD 279	-6.27	-6.6
ACD 68	-4.93	-4.6	ACD 293	-	-2.7
ACD 72	-	-6.1	ACD 297	-	-6.1
ACD 74	-4.93	-4.6	ACD 302	-6.27	-6.6
ACD 81	-	-9.4	ACD 307	-	-6.9
ACD 100	-	-9.3	ACD 309	-	-5.6
ACD 133	-7.23	-7.5	ACD 311	-	-5.5
ACD 150	-	-6.5	ACD 315	-6.74	-7.2
ACD 152	-7.71	-8.8	ACD 327	-	-4.0
ACD 167	-8.70	-8.7	ACD 332	-	-6.3
ACD 180	-6.02	-6.6	ACD 334	-	-6.1
ACD 183	-	-5.5	ACD 335	-6.28	-7.8
ACD 186	-	-12.5	ACD 345	-	-4.5
ACD 193	-11.44	-12.6	ACD 346	-4.35	-6.9
ACD 196	-	-4.4	ACD 356	-	-6.9
ACD 198	-	-7.1	ACD 357	-	-6.0
ACD 207	-	-6.6	ACD 389	-	-3.7
ACD 213	-	-6.2	ACD average	-	-6.3
ACD 218	-	-6.0	*L. multiflorum*	-6.36	-7.2
ACD 219	-	< -14.0	*F. arundinacea*	-11.37	-12.0
ACD 249	-	-4.5	*Lm/Fa*	-9.37	-11.0
ACD 253	-	-3.5			

Probit analysis is considered more accurate than the graphical method for estimating the LT_{50} value. However probit analysis can be employed only when there are sufficient intermediate responses to freezing. The freezing tolerance experiment described here required the use of as many different ACD plants as possible to assess the extent of genetic variation available for freezing tolerance within the androgenic population. In order to include a substantial number of androgenic genotypes in the test procedure, it was necessary to limit replicates to 5 for each temperature treatment. In future, it will be necessary to increase the number of replicate tillers per temperature treatment to improve accurate determination of LT_{50} for each ACD plant. Increased numbers of plants with intermediate temperature treatment response will further assist in assigning an accurate LT_{50} to individual plants. In many cases (ACD plants No. 6, 9, 45, 56, 72, 150, 183, 196, 207, 213, 218, 249, 293, 297, 309, 311, 327, 332, 334, 345, 356, 357 and 389) there were insufficient replicate data or alternatively insufficient intermediate response data (in the cases of ACD plants No. 81, 100, 186, 198, 219, 255, 257 and 307) to calculate the LT_{50} by probit analysis.

Figure 5.2 describes ACD plants where sufficient intermediate freezing tolerance response data existed to compare LT_{50} calculated by probit analysis and by graphical means. Although there were some small differences between the two methods, these differences were not large enough to be significant, and thus an overall assessment of freezing tolerance by graphical means was considered sufficiently accurate to compare freezing tolerance of all ACD plants. The overall LT_{50} was estimated by graphical means. The data is supported by that from the *Lolium* and *Festuca* controls which as expected, had contrasting freezing tolerance with the *Lolium* more cold sensitive than the *Festuca*. The pentaploid hybrid was intermediate between the parental species for freezing tolerance but had only slight inferior freezing tolerance compared with the *Festuca* parent.

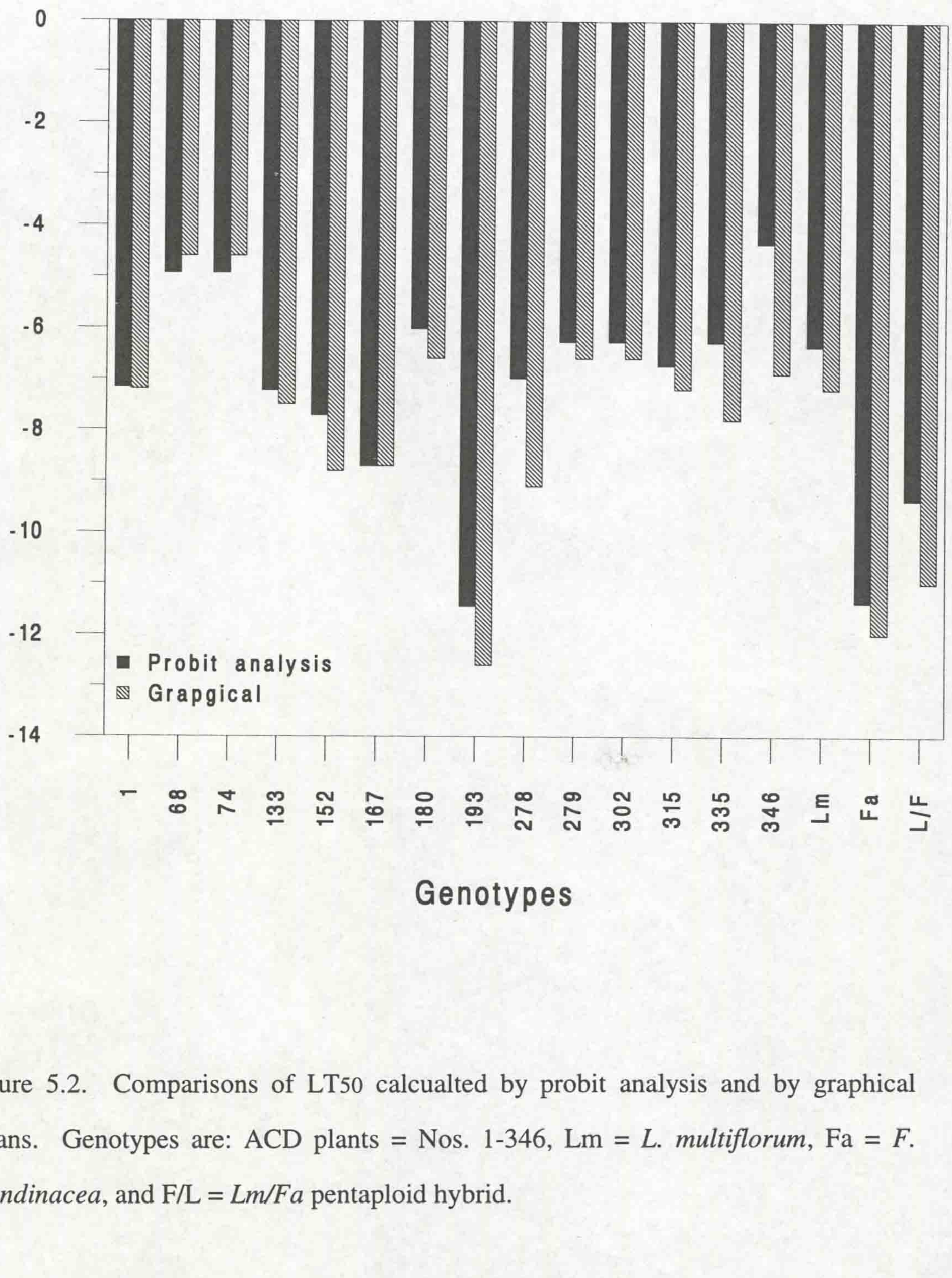


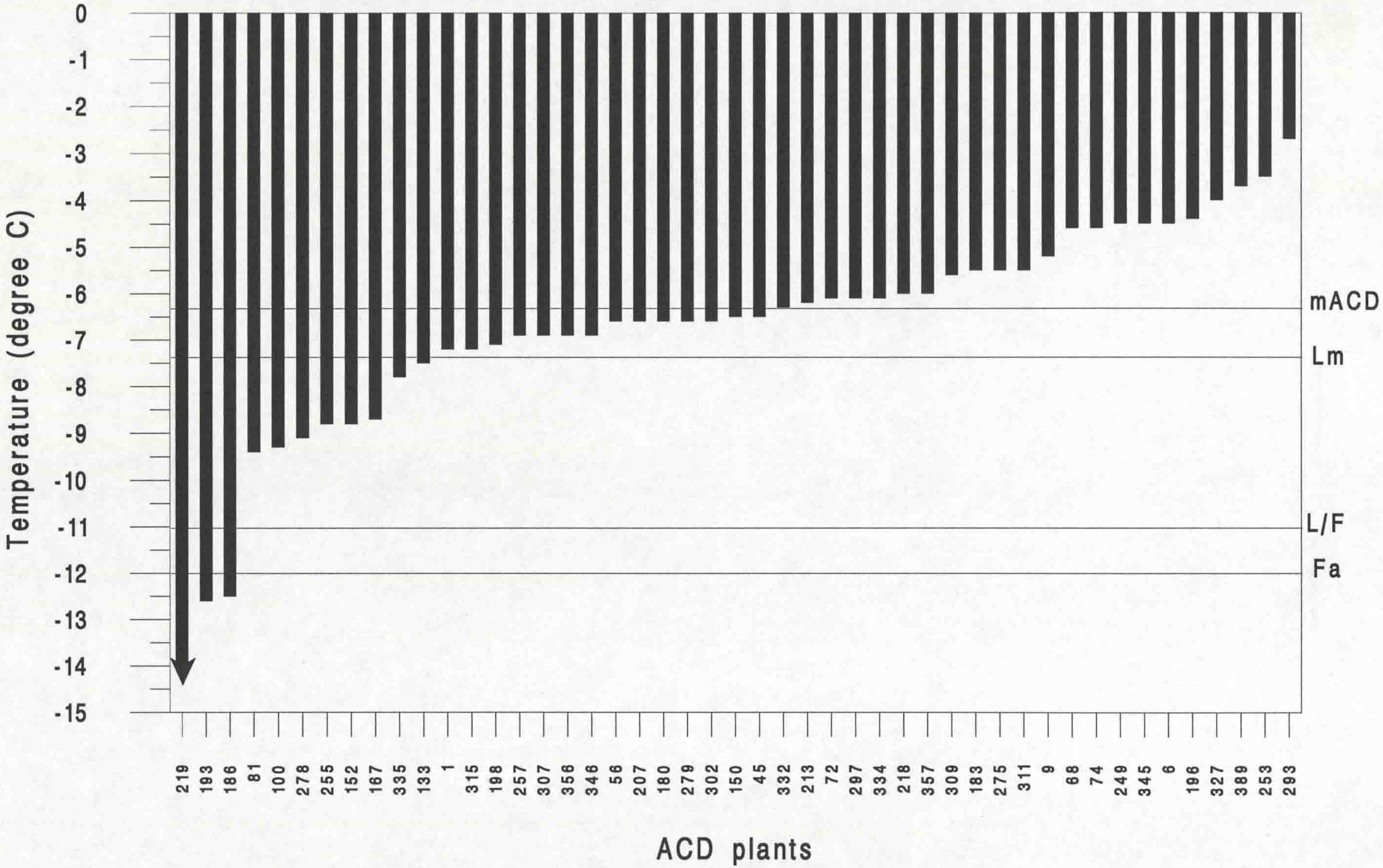
Figure 5.2. Comparisons of LT50 calcualted by probit analysis and by graphical means. Genotypes are: ACD plants = Nos. 1-346, Lm = *L. multiflorum*, Fa = *F. arundinacea*, and F/L = *Lm/Fa* pentaploid hybrid.

Figure 5.3 illustrates the range of LT_{50} between ACD plants. The range was from -2.7°C in plant No. 293 (most freezing susceptible) to ACD plant 219 (100% survival at -14°C). Two other ACD plants (No. 186 and 193) were more freezing tolerant than the tall fescue control (LT_{50} = -12.0°C).

Cold tolerance is considered a complex quantitative character and controlled by many genes (e.g. Sutka, 1981; Norell *et al.*, 1986; Sutka, Veisz and Kovacs, 1986; Limin and Fowler, 1988 and Larsen, 1994). The genetic diversity revealed by anther culture in this study is evidence that the technique provides an effective means to select genotypes which carry genes involved in determining freezing tolerance. Opportunities may have arisen in the polyhaploid state for expression of relevant recessive genes that affect response to stress through freezing. Opportunities to select and achieve expression of such gene combinations by conventional plant breeding by either forming amphiploids or by back crossing is very limited and maybe impossible. Unless recessive genes are tagged by molecular genetic markers to enable their selection, their selection would be impossible by conventional breeding methods.

Figure 5.3 shows a total of 36 ACD plants with the same or lower freezing tolerance as that of *L. multiflorum*. Although overall the range of cold resistance of ACD plants was very wide (with extremes from LT_{50} = -2.7°C in ACD plant 293 to < -14.0°C in ACD plant 219) the LT_{50} of most ACD plants approximated to that of *L. multiflorum*. A total of 8 ACD plants had cold resistance intermediate with *L. multiflorum* and *F. arundinacea*. However 3 ACD plants (No. 186, 193 and 219) were more cold resistant than either parental species. Since only 47 ACD plants have thus far been assessed for freezing tolerance, the extent of variation for this trait among the ACD plants may be even greater. However, the ACD plants selected for the freezing tolerance experiment were randomly selected and were considered to include representatives covering the entire range of obvious morphological differences. Thus the plants tested were considered to be a representative sample.

Figure 5.3. LT_{50} for low temperature tolerance of ACD plants. Genotypes are: ACD plants = Nos. 1-389, Lm = *L. multiflorum*, Fa = *F. arundinacea*, and F/L = *Lm/Fm* pentaploid hybrid and mACD = mean LT_{50} of the ACD plants.


0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
-12
-13
-14
-15
Temperature (degree C)
mACD
Lm
L/F
Fa
219
193
186
81
100
278
255
152
167
335
133
1
315
198
257
307
356
346
56
207
180
279
302
150
45
332
213
72
297
334
218
357
309
183
275
311
9
68
74
249
345
6
196
327
389
253
293
ACD plants

4811

The number of ACD plants with superior tolerance to freezing stress than *F. arundinacea* or the pentaploid parent were found at a frequency of circa 6%. Since these plants represent the products of meiosis in the pentaploid hybrid, the frequency of genotypes with freezing tolerance greater than the hybrid or *Festuca* parent represented the potential of that hybrid to produce gene combinations which offer superior freezing tolerance above expectations based on parental performance. Presumably these gene combinations will be in the hemizygous state and can be made homozygous and fixed by chromosome doubling. The realised potential for selecting genotypes with increased stress tolerance above that found in the parental hybrid illustrates well the value of anther culture.

Most plants from temperate, arctic and alpine climates have the ability to cold harden when exposed to short days and decreasing temperature (above zero) during the autumn (Öquist, 1982). Among the general conclusions to have emerged are that the ability to cold acclimate is a quantitative genetic trait (Humphreys and Eagles, 1988 and Thomashow, 1993). The increase in freezing tolerance that occurs during cold acclimation includes change in membrane cryobehaviour (Thomashow, 1993). The plasma membrane of the plant cell is considered to be a primary site of damage during a freezing-thaw cycle. Anther culture derived plants and their control genotypes were cold acclimated at +2°C temperature (day and night) and 8 hours day length for two weeks. Cold acclimation will allow differentiation between chilling-sensitive and chilling tolerant genotypes. In fact, no androgenic plant showed any obvious deleterious effects from the cold hardening treatment prior to freezing, and all cold acclimated plants were used in the freezing treatment.

ACD plants represented a vast array of cold tolerance variation. ACD plants 219, 193 and 186 with freezing tolerance superior to the *Festuca* control demonstrate that selections made under quite high selective pressure could yield genotypes with freezing tolerance greater than the parental *Lm/Fa* pentaploid hybrid. Seventy percent of ACD plants were not as tolerant to freezing temperatures as the *L. multiflorum* control. This may be due to combinations of deleterious gene(s) which reduce plant

vigour despite the presence of genes for freezing tolerance. Alternatively, *F. arundinacea* chromosome and genetic imbalance which must occur in androgenic plants will likely disturb important metabolic pathways involved in freezing tolerance and might be another reason why many androgenic plants have inferior freezing tolerance even to the *Lolium* control. More than 23% (11 ACD plants) had better cold tolerance than *L. multiflorum*

Improved survival of hardened plants at lower freezing temperatures is not attributed to improved hardiness of the main apex but depends on the ability to establish viable re-growth from lateral buds during the recovery period (Eagles Williams and Louis, 1993). Although the re-growth of tillers from lateral buds was not recorded in the present study, it was observed that in most cases and especially under the lowest temperatures, the main apex was killed and recovery was from tillers produced from lateral buds.

The LT_{50} of *F. arundinacea* showed that the fescue was almost as cold tolerant as the best cultivars (cvs. Hawk and Aber valley) of *F. rubra*, a species known for high freezing tolerance and native to countries such as Norway (McHugh, 1986). The future use of the ACD plants 219, 193 and 186, which compare well with the best cultivars of *F. rubra*, for use in breeding programmes to produce novel cold tolerant cultivars, appears an attractive prospect.

A strong negative correlation between growth at low temperatures and cold hardiness was reported for Mediterranean forage grasses types (Eagles and Fuller, 1982). Although no measurements were made of growth under low temperatures by the androgenic population, in summer field conditions, the three most cold tolerant ACD plants (Nos. 219, 193 and 186) demonstrate particularly high growth rate (see chapter 4). While no assessments have been made on leaf quality characters such as digestibility etc., the combination of extreme cold tolerance and high growth rate found in the three androgenic plants would support the need for further investigations of the androgenic plants as potential germplasm for development and exploitation as fodder in Northern latitudes.

CHAPTER SIX

THE EFFECT OF DROUGHT STRESS ON *L. MULTIFLORUM* × *F. ARUNDINACEA* PENTAPLOID ANTHER CULTURE DERIVED PLANTS

6.1. Introduction

"Drought", which is derived from an Anglo-Saxon word meaning dry land, is a comparative not an exact term (Hulse, 1989). In meteorological terms, drought is commonly defined as a period without significant rainfall (Turner, 1979). Drought stress is defined as a water stress due to lack of rainfall, or more simply a natural water stress as opposed to an artificial water stress (Levitt, 1980). In agriculture drought is defined as an unbroken dry period that results in a loss of yield below that expected under optimal water supply. It is the major environmental constraint to world agriculture and limits plant growth and productivity (Boyer, 1982; McWilliam, 1986; Smith and Griffiths, 1993). The extent of world drought is difficult to estimate since most regions receive inadequate rainfall at least sometime during the growing season (Nabors, 1983). Raheja (1966) maintains that 35% of crop land receives less than 760 mm of precipitation per year and much of the remaining 65% suffers drought sometimes during the growing season. Acevedo and Fereres (1993) pointed out that drought and other stresses such as water-logging, salinity, low mineral nutrients, or extremes of temperature, pH, or metal deposits, are common throughout the world so that it is very difficult to find stress-free areas where crops may approach their potential yields. It has been estimated for example that, because of physicochemical stress, the yield of field crops in the United States is only 22% of the genetic potential yield (Boyer 1982). Arid and semi-arid regions, which are usually characterized by drought, extreme temperatures and saline soil and water, constitute about 40% of the world's land surface (Fischer and Turner, 1978). These limitations will increase in severity for world agriculture as a result of rising population levels (Smith and Griffiths 1993).

Drought stress might operate on the plant directly or indirectly. It has been suggested that it may act directly by mechanisms involving a reduction in the chemical potential of water, through a reduction in hydrostatic pressure in the cells (Shalhevet, 1993). Alternatively, stress from water deficit, can act indirectly, such

as through a decrease in protein content and the incorporation of amino-acids into protein by increased ribonuclease activity and dissociation of polyribosomes (Galiba *et al.*, 1989).

Although arid and semiarid areas suffer more from drought, availability of water is perhaps of equal importance in temperate zones by its impact on yield of crops that are less adapted to severe drought (Christiansen, 1979). The irregular distribution of rainfall leads to periods in which water availability limits growth (Boyer, 1982). Even in the relatively wet maritime climate of England and Wales, shortage of water during the growing season is the main cause of year-to-year variability in yield of herbage (Morrison *et al.*, 1980)

Some higher plants can cope with drought by being drought resistant. Some plants in arid areas have developed an avoidance strategy (Mohr and Schopfer, 1995). These are usually short-lived species which escape from having to withstand drought by their short growth cycle which is completed within the rainy season. They survive the drought period in the form of desiccation tolerant seeds (Mohr and Schopfer, 1995). However, genotypes with very short life cycle, and low yields are not as good (in wet conditions) as are most temperate forage grasses. For example Thomas (1997) stated that accessions that come from parts of Europe with a high summer insulation and only small soil water deficit would be more useful to increase drought tolerance of temperate grasslands compared with those from drier climates which have slow growth rates and are therefore agronomically unacceptable in the U.K.

The perennial grasses from a dry climate and high temperature may show slow or no growth during a summer drought. For example under various levels of water deficit no growth was observed in the summer in the drought resistant temperate forage grass cocksfoot (Volaire, 1994 and Norris and Thomas, 1982). Seedlings of grasses such as bromegrasses and corn that appear to be highly tolerant of heat just after sowing, pass through a stage of rapid growth when they are sensitive to heat injury, and then become increasingly heat tolerant as they become

older (Schultz and Hayes, 1938; McAlister, 1944; Laude and Chaugule, 1953 and Wardlaw and Wiliams, 1966).

In many temperate grass species such as *Phalaris tuberosa,* survival of extreme summer drought is aided by the induction of dormancy or by the formation of corms (Evans, Wardlaw and Wiliams, 1966). Rewatering after stress may lead to regrowth. Perennial grasses such as *Lolium* and *Festuca* will flower during the summer and at this stage are more sensitive to drought (Thomas and Evans, 1990). In dry conditions, they may complete flowering and set seed earlier than in non water-deficit conditions. As a result grasses show increasingly early heading from north to south in Europe (Lorenzetti *et al.*, 1971). For example even within one region of Spain *Dactylis glomerata* populations which were exposed to drought flowered several weeks before those that were in more sheltered areas (Lumaret *et al.*, 1987). There is also variation between temperate forage grass species for drought resistance. The ranking in decreasing order of drought resistance are *Bromus madritensis* > *Dactylis glomerata* > *F. arundinacea* > *L. perenne* > *Phleum pratense* > *L. multiflorum* > *Poa trivialis* (Garwood *et al.*, 1979; and Norris and Thomas, 1982).

Hall (1993) defined drought resistance as the average yield or survival of a genotype compared with other genotypes subjected to the same drought, and where drought escape is not a major factor. Whilst this definition is adequate for annual cereals grown in temperate regions, it is less applicable to perennial forage crops which are harvested several times a year. This is especially so in seasonally dry (e.g. Mediterranean) climates, where persistence and survival rather than herbage yield during drought are more agronomically desirable. This definition of drought resistance involved genotype comparison and is therefore, consistent with the definition of plant adaptation. Drought susceptibility, in contrast, has been defined as the relative yield of a plant under drought compared with its performance under well-watered conditions (Fischer and Maurer, 1978). Drought susceptibility defined in this way is not directly useful for assessing the adaptation of a genotype in either

an agricultural or an ecological context (Fischer and Maurer, 1978). A drought-susceptible genotype, according to this definition, would simply have a relatively low yield under drought compared with its yield under well-watered condition. It could also have either a higher or a lower absolute yield under drought than other genotypes and, consequently, either higher or lower drought resistance. The value of measures of drought susceptibility is that they provide estimates of the strain due to drought stress, and may be more effective than drought resistance when comparing the drought adaptation of different species that have substantial differences in yield potential (Hall, 1993).

Plants of many species respond to drought by increasing the production of assimilates directed into root growth, and thus increasing the root : shoot ratio and the volume of soil water available to the plant (O'Toole and Bland, 1987). Many root characters affect water uptake, and include total mass, distribution, maximum depth explored, branching, diameter, root hairs, and possibly infection by symbiotic mycorrhizae (Thomas, 1997). A deep root system is exhibited by *F. arundinacea* Schreb (Garwood and Sinclair, 1979). In general, it seems that only a few deep roots may be needed to supply water to a droughted plant (McWilliam and Kramer, 1968; Sharp and Davies, 1985 and Passioura, 1988). The effectiveness of roots at taking up water seems to decline with distance from the tip (Hansen, 1974) and it may be that newly produced roots will be more effective than ones formed earlier. Of the other root characters that may play an important role in drought resistance in *F. arundinacea,* is root diameter. Genetic variation in roots was positively correlated with drought resistance in tall fescue (Torbert *et al.,* 1990). Jupp and Newman (1987), and Salter *et al.* (1984) reported in *L. multiflorum* that there was an increase in root density under drought conditions. In contrast, other researchers found drought reduced root density in maize, beet, and wheat (e.g. Sharp and Davis, 1985; Brown and Biscoe, 1987 and Narayan and Misra ,1989).

Hybrids between *L. multiflorum* and *F. arundinacea* (Lewis, 1966 and Buckner *et al.*, 1977) generally are intermediate between their parents in most

characteristics being more hardy than *L. multiflorum,* but also less digestible. Comparisons between these two species in a controlled environment (Thomas, 1994) showed that the tall fescue has a higher leaf extension rate under stress, higher stomatal conductance on both leaf surfaces, higher modules of elasticity, and possibly higher leaf extensibility and hydraulic conductance.

It has been shown, using phosphoglucoisomerase (PGI/2) as a genetic marker, that it is possible to transfer genes from *F. arundinacea* into diploid *L. multiflorum* using the same pentaploid *L. multiflorum* (4x) × *F. arundinacea* (6x) hybrid used here for androgenesis (Humphreys, 1989). This novel introgression programme has been effective in transferring drought resistance from *F. arundinacea* into the drought susceptible *L. multiflorum* (Humphreys and Thomas, 1993). Although in field drought trials, backcross populations were on average less drought resistant than the *L. multiflorum,* 3% of the backcross derivatives were as drought resistant as *F. arundinacea*.

It was considered likely that the androgenic population would also show a range of drought tolerance at least equal to that exhibited by the *Lolium* and *Festuca* parent *spp*. The aim of the work described here was to assess the range of drought resistance expression in the polyhaploid plants derived from anthers of a single *L. multiflorum* × *F. arundinacea* pentaploid hybrid (see chapter 3), and to compare the drought resistance of androgenic plants with the pentaploid hybrid and its parental species, i.e. *L. multiflorum* and *F. arundinacea*.

6.2. Materials and methods

6.2.1. Plant material and the experimental design for assessing drought resistance using a rain-out shelter in field conditions

As explained in Chapter two (Section 2.3) the aim of the field trial was to assess drought resistance in anther culture derived (ACD) plants from the *L. multiflorum* × *F. arundinacea* pentaploid hybrid using a polythene rain-out shelter under field conditions. A total of 88 ACD plants which display wide variation in morphology, four genotypes of *L. multiflorum* cultivar 'Tribune' (as drought sensitive parental species), four genotypes of *F. arundinacea* Bn 949 (as drought resistant parental species), and four ramets of the original *L. multiflorum* × *F. arundinacea* pentaploid hybrid, were selected for inclusion in the field drought experiment.

All 100 plants selected for use in the field experiment were separated into five equal clonal parts and prior to planting in the field were maintained in pots in an un-heated glasshouse and allowed to establish. Four replicate clones of each genotype were transferred to the field in a randomized block experimental design (main fields = environment, sub fields = genotypes) on 15 May 1994 at the Institute of Grassland and Environmental Research (IGER), Aberystwyth, UK. The two blocks (droughted and irrigated control) were located in the field about five meters apart. One barrier row of parental populations as guard plants was grown around each block. The distance between plants was 30 cm between rows, and 20 cm within rows. No fertilizer was supplied. All replications were irrigated as required to allow plants to properly establish (completed 6 June 1994).

Prior to flowering, one block consisting of two adjacent replicates (I and II) was covered by a mobile polythene rain-out shelter and used for assessing drought resistance (Figure 6.1a). The irrigation was continued on the remaining replicates (III and IV) as often as necessary to ensure no water stress.

The herbage was cut and harvested to a height of about 6 cm above the ground on 4 occasions during the experiment and dried in an oven in brown paper bags for 24 hours at 80°C. The fresh and dry weight of herbage of each genotype was recorded for each replication. The dates of cutting are given in Table 6.1.

Table 6.1. Phases of drought treatment

Cuts	Date	Experimental period
0	06. 06. 1994	Commence drought experiment
1	11. 06. 1994	Prior to onset drought
2	06. 09. 1994	After 87 days drought treatment
3	24. 09. 1994	After 105 days drought treatment
4	07. 11. 1994	Recovery period (44 days irrigation)

Tiller number per genotype was determined for all replicates at three stages during the drought experiment. Initial tiller number (T0) was determined when genotypes were split into 5 equal clonal parts (25th March 1994) prior to establishment in pots in the glasshouse. Tiller number was subsequently determined in the field at Cut 0 (T1) (6th June, following establishment in the field but prior to beginning of drought experiment) and finally at Cut 3 (T2) (24th October, at the end of the drought treatment following 110 days of drought).

Figure 6.1. Drought experiments a) Mobile polythene rain-out shelter under field conditions and b) glasshouse used for assessing drought resistance in ACD plants of pentaploid hybrid.

6.2.2. Plant material and experimental design for assessment of drought resistance under glasshouse conditions

A glasshouse experiment (Figure 6.1b) was designed so as to minimise interference (described in section 6.4.1.1) from variables such as soil, light, and temperature which differed between the irrigated and droughted plots in field trials and which together, might bring error, into interpretations of the response of ÅCD plants to drought. In particular, consideration was given to differences in natural vigour between genotypes and the potential for differences in plant size affecting interpretations of levels of drought tolerance. For this reason care was taken that all plants commencing the drought experiment were the same size (i.e. same number of tillers) and that plants were regularly cut to prevent over-shading of the slow-growing genotypes by those with faster growth rates and greater foliage.

Fifty seven ACD plants together with the 12 parental controls described previously for the field experiment (Section 6.2.1), and a further 71 ACD plants were used for the glasshouse drought experiment. Ten single tillers from each genotype were isolated and grown separately in multitray-pots in an unheated glasshouse.

Ten brick bins with dimensions approximately 150 cm (length) × 100 cm (width) × 100 (height) were each filled with homogeneous soil mixture containing John Innes No. 3 potting compost. The bins were located in rows, 5 to the North and 5 to the South of a single-ridge cold glasshouse. Bins were irrigated with hose-pipes with very fine nozzles to soak through the soil uniformly and thoroughly. The brick-bin walls were covered with polythene sheets to prevent water seepage through the walls from bins used as irrigated controls into neighbouring bins used for the drought experiment.

All 10 bins contained 140 identical genotypes of equal size (i.e. there were 10 replicates), planted in each bin in a different randomized pattern on 7 June 1995. Two barrier rows of three Italian ryegrass varieties (cvs.Titania, Trident and Tribune

(All 2n = 2x = 14)) were planted as guard plants around each bin. The distance between plants both between and within rows was 8 cm. No additional fertilizer was supplied during the duration of the experiment. All bins were irrigated daily for 17 days to allow genotypes to establish. Subsequent to establishment, 8 replicate bins were droughted for 77 days. Two replicate bins, one on the North and the other on the South side of the glasshouse were irrigated thoroughly on a daily basis as controls. All plants were cut as frequently (normally weekly) as necessary to maintain a maximum height of about 6 cm above the soil surface to prevent shading and competition for light. All plants were cut at the same time during the experiment to ensure that all treatments were true replicates. The stages of the glasshouse drought experiment are summarised in Table 6.2.

Table 6.2. Phases of glasshouse drought experiment

Date	Experimental activity
21-27. 04. 1995	Isolate tillers and plant in multitrays in an unheated glasshouse
8-9. 06. 1995	Plant small plants in brick bins in a completely randomized experimental design
26. 06. 1995	Commence drought experiment
10. 09. 1995	Record amount of green leaf at the end of the drought period
13. 10. 1995	Record amount of green leaf after recovery following drought

At the end of the drought period and also following 33 days recovery and daily irrigation at the completion of the drought experiment, the condition of each plant, irrespective of size and without reference to plant identify, was recorded using a scale of 7 criteria (indicated by 0, 2, 5, 8, 11, 14 and 17, Table 6.3) and sub-scales for each of the main groups of criteria (excepting the first group) designated 1, 3, 4, 6, 7, 9, 10, 12, 13, 15, 16 and 18 as described in Table 6.3. The main criteria (0, 2,

5, 8, 11 and 14) described surviving green leaves after drought in accordance with the scale 0-5 used by Humphreys and Thomas (1993). In this study, in order to identify more precisely differences in survival between genotypes, the scale unit of drought survival as described by Humphreys and Thomas was further sub-divided into three sub-scale units. By separating the scale into 18 distinct component, genotypes could be compared statistically, using analysis of variance.

Table 6.3. Criteria and definitions used to describe tiller survival in the glasshouse drought experiment.

Scales	Definition
0	Completely dead, no green material visible, even when tillers dissected.
1	Lower extreme of scale 2.
2	Trace of green tissue, usually at the base of the youngest leaves
3	Upper extreme of scale 2.
4	Lower extreme of scale 5.
5	Large amount of dead tissue, but some tillers having at least one green lamina.
6	Upper extreme of scale 5.
7	Lower extreme of scale 8.
8	Approximately half of the tillers with appreciable amounts of green leaf lamina.
9	Upper extreme of scale 8.
10	Lower extreme of scale 11.
11	Most or all of the tillers alive, but with most leaves scorched at the tips.
12	Upper extreme of scale 11.
13	Lower extreme of scale 14
14	All tillers alive, and with little or no scorching and including those in recovery which were able to produce as many new tillers as were lost.
15	Upper extreme of scale 14
16	Lower extreme of scale 17
17	More new tillers produced during the recovery period.
18	Upper extreme of scale 17

6.3. Analysis of data

6.3.1. Field experiment

Analysis of variance was used to assess variation in dry herbage mass and tiller production according to genotype, and the effect of environments in which plants were grown: environment I (drought) and environment II (irrigated). Because of the constraints of the physical layout of the experiment in the field, it was not possible to combine the two environments in the same analyses of variance. Analyses of variance were calculated using GLM (General Linear Models procedure) SAS (Statistical Analysis System, 1990). Since the genotypes varied greatly in size etc., homogeneity of error of variance was achieved by transforming the data as $\log_{10}(x + 1)$. The number of tillers recorded (T0 and T1) and the dry herbage mass at Cut 1 (11th June, prior to the imposing of the drought treatment) were tested as possible covariates of dry herbage mass. This was done to account for initial differences in natural vigour and plant size among genotypes. Tiller numbers (T0 and T1) were not found to be significant covariates whereas dry herbage mass at Cut 1 was highly significant ($P \leq 0.001$). Thus dry herbage mass at Cut 1 was considered as a covariate of dry herbage mass. Also, the number of planted tillers (T0) and the tiller number at the start of the drought experiment (T1) were tested as a covariate for tiller production during the drought period. Only tiller number at the start of the drought experiment (T1) was highly significant ($P \leq 0.001$), thus it was considered as a covariate of tiller production during the drought at the end of drought experiment (Cut 3). The effect of introducing the above into the overall comparative analysis of drought tolerance between different anther culture derived plants substantially reduced error due to variation in initial plant size.

Pairwise least significant differences (LSD) were calculated according to the Fisher method. [This does not explicitly control the experimental error rate in

comparing treatment means (Maxwell and Delaney, 1990), and conclusions must be drawn, conservatively.]

6.3.2. Glasshouse experiment

Analysis of variance was used to compare survival of each ACD plant and control genotypes, the effect of the two environments, droughted (E1) and irrigated (E2), and also to take account of the North and South orientation of the brick-bins in the glasshouse. The approach is as described in Chapter 4.

6.4. Results and discussion

6.4.1. Field experimental results and discussion

If the major objective of the breeding programme is to select for greater resistance to low-rainfall drought environments, selection should be undertaken in such environments (Baker, 1989). Thomas (1997) stressed that the most reliable and productive forage grasses in a particular environment are those adapted, by natural selection or breeding, to slightly harsher conditions. This section describes an experiment designed to determine the range of drought resistance of ACD (anther culture derived) plants under field conditions at IGER in 1994. A rain-out shelter was used to simulate approximately natural drought conditions for 110 days during the growing season. Since drought is a comparative term (Hulse, 1989) irrigated trials were used as control. The pentaploid hybrid expressed excellent drought resistance under field conditions. None of the genotypes (except No. 219 at Cut 2) yielded more than the parent hybrid. However the mean yield of only 19.3% (Cut 2, early drought) and 2.3% (Cut 3, end of drought) of the ACD plants were not statistically significant different from pentaploid hybrid.

Shoot biomass in forage grass breeding is important from two points of view. Firstly, the leaf is directly involved with production of assimilate for growth and yield (Blum, 1988). A large part of the biomass of forage grass is leaf. Also, the stems of forage grasses contain chlorophyll and to some extent can be involved in production of assimilate of growth.

Secondly, unlike other crops grown for their grain or vegetative parts, grass and grassland products are used almost entirely for the feeding of ruminant animals (Jones and Lazenby, 1988).

6.4.1.1. Field test

The field areas used for the drought and irrigation treatment were very close (separated by only a few meters from each other). In order to determine the homogeneity of the two field areas, dry herbage mass of control genotypes (*L. multiflorum*, *F. arundinacea* and the *L. multiflorum* × *F. arundinacea* pentaploid hybrid) was compared between sites at Cut 1 (prior to the onset of drought treatment).

Analysis of variance of dry herbage mass per plant (g) of the control genotypes at Cut 1 is presented in Appendix 6.1. Dry herbage mass in the controls was significantly affected by field location ($P = 0.03$). Mean dry herbage mass in field I (plot for droughting) (39.5 g) was two fold higher than in field II (plot for regular irrigation) (18.7 g). Although between sites, the genotype dry weight was different, genotype × environment interaction, was not significant. Figure 6.2 shows all three control genotypes had higher yield in field I (nominated for drought) than field II (nominated for use as the irrigated control). Each genotype was represented by clones at both field locations and differences in dry herbage mass between clones at the two field locations is explained in part by difference in soil conditions (texture and fertility), but also by the advantageous growth conditions (e.g. higher temperature, lower wind stress etc.) which were initially present under the rain out-

shelter prior to the onset of drought stress. Since conditions for growth in field II (irrigated control) may be inferior to those in field I, differences in growth rate between replicates under drought stress and under irrigation at the end of the drought experiment may be an underestimation of the real effect of drought on plant growth.

6.4.1.2. Dry herbage mass

Analysis of variance of log-transferred ($\log_{10}$ (x + 1) herbage mass dry matter per plant after 92 days of drought (herbage weight after 87 days with the use of the yield at Cut 1 as a covariate) of ACD plants and of the control genotypes is presented in Appendix 6.2. Genotype and environment caused significant difference in herbage yield but there was no significant genotype × environment interaction. As shown in Figure 6.3 only genotype 219 in the irrigated environment yielded significantly more herbage than *L. multiflorum*, this value being similar to that of *F. arundinacea.* The pentaploid hybrid yielded similarly to *F. arundinacea* in both environments. Although high yielding genotypes 219 and 193 out-yielded both *Lolium* and *Festuca* species, and plants Nos. 133, 186 and 356 gave a better yield than *L. multiflorum*, none of these differences were significant.

Mean dry herbage mass of genotypes in the irrigated environment (2.97 g) was two fold higher ($P \leq 0.01$) than in the droughted environment (1.49 g). Figure 6.3 shows 25 ACD plants (219, 186, 334, 180, 72, 192, 218, 345, 309, 196, 193, 167, 255, 100, 74, 81, 315, 102, 1, 341, 93, 104, 197 and 253) with significantly higher yield under the irrigated conditions compared with the droughted environment ($P \leq 0.05$). Although no ACD plant yielded significantly more in the droughted environment compared with the irrigated environment, 13 genotypes (including 360, 335, 84 and 150) were slightly higher yielding under drought. The control genotypes yielded significantly more in the irrigated compared with the droughted environment.

Analysis of variance of log-transferred yield produced during the 18 days from Cut 2 to the end of the drought experiment (Cut 3) is presented in Appendix 6.3. Genotype and environment effects and their interactions were again significant sources of variation. Figure 6.4 illustrates that in the droughted environment 10 ACD plants yielded more than *L. multiflorum* (plants 219, 193, 186 and 327 were significantly greater ($P \leq 0.01$), and 4 plants (219, 193, 186 and 133) yielded more than *F. arundinacea* with plants 219 and 193 statistically significant ($P \leq 0.01$). In the irrigated environment, only 3 genotypes (219, 193 and 133) produced significantly more herbage than both tall fescue and Italian ryegrass. Under drought, tall fescue yielded significantly more than Italian ryegrass but there was no significant difference in yield under irrigation. None of the genotypes in either environment, out-yielded the pentaploid hybrid. However the yield of the pentaploid hybrid was not significantly greater ($P \leq 0.05$) than genotypes 219 and 193 in either environment nor genotype 133 in the irrigated environment.

Mean dry herbage mass of genotypes in the irrigated environment (0.43 g) was higher ($P \leq 0.0001$) than in the droughted environment (0.27 g). A total of 10 ACD plants (219, 133, 100, 74, 334, 278, 307, 104 and 180) and the pentaploid hybrid yielded more under the irrigated conditions than in the droughted environment, and 4 plants (327, 360, 335, and 357) showed the reverse response. Most ACD plants (e.g. 193, 299, 109 and 260) showed similar yield in both environments. Plants 70, 253, 150, 95, 293, 249, 332, 180 and 297 were particularly sensitive, and died under drought (Figure 6.4).

Analysis of variance of herbage dry matter after recovery for 44 days following drought is given in Appendix 6.4. Genotype, environment and their interaction were again significant. Figure 6.5 illustrates recovery after drought with 8 ACD plants having yielded more than both *L. multiflorum* and *F. arundinacea*, comparisons being highly significant in the case of plants 219, 193, 186 and 133 in the droughted plot ($P \leq 0.001$). In the irrigated environment none of the ACD plants or *F. arundinacea* yielded more dry herbage mass than Italian ryegrass but a total of

8 plants (especially 193, 186 and 133) had improved growth rate over tall fescue ($P \leq 0.01$). Italian ryegrass yielded significantly more in the irrigated than in the droughted environment but differences between treatments were not significant for tall fescue. The pentaploid hybrid had a higher herbage mass ($P \leq 0.01$) than all other genotypes in both environments.

Mean dry herbage mass of plants under continuous irrigation (0.13 g) exceeded ($P \leq 0.01$) the mean dry herbage mass in clones in recovery following drought (0.09 g). The effects of drought had confounded the initial advantages (described earlier in this Chapter) which gave rise to increased growth under the rain-out shelter prior to obvious signs of stress from water deficiency. The analysis indicated a significant genotype x environment interaction. Plant 219 yielded much more in the droughted environment than in the irrigated environment whereas genotypes 193, 186, 133, 100 and 180 yielded significantly more in the irrigated environment than in the droughted environment. However, the dry herbage mass of many genotypes was not significantly different in the two environments. Fourteen ACD plants (e.g. Nos. 177, 95, 150 and 249), presumably with low growth rate and natural vigour failed to establish either in the droughted or irrigated field-plots and died in both environments. A few other plants, low in vigour and mainly producing low dry herbage matter, succumbed completely to stress by drought e.g. Nos. 357, 192, 278 and 104. Growth of Italian ryegrass and the pentaploid hybrid at Cut 4 was significantly greater under the irrigated control conditions ($P \leq 0.01$). Despite growth of tall fescue in the control exceeding that found in the drought recovery treatment, this was not significant.

Figure 6.6 compares plant growth rate early and late in drought. The genotypes that had below average growth-rate during early drought also grew very slowly during late drought, with the exception of genotype 205 which increased in growth rate during the drought treatment. The genotypes with intermediate growth rate during early drought varied widely in growth during late drought. Some genotypes grew rapidly prior to Cut 2 but produced nothing or very low herbage

mass in Cut 3 e.g. ACD plants Nos. 307 and 357. Other genotypes which showed little growth prior to Cut 2, decreased further in growth rate in Cut 3 e.g. No. 205. Many genotypes which had a similar yield at Cut 2 had a big gap in growth rate by cut 3 e.g. ACD plants 327 and 167 (drought tolerant) in comparison with plants 255 and 356 (drought sensitive). In many cases it was not possible to predict the yield of Cut 3 by the yield of Cut 2 because many plants with good growth up to Cut 2 decreased in growth rate by Cut 3 as the drought stress took effect e.g. ACD plants 327 and 167 with 356, and 315 with 357 and 307. However, some high yielding genotypes at Cut 2 maintained their high growth rate to Cut 3 e.g. Nos. 219, 193, 186, 133 and *F. arundinacea*. The majority of ACD plants showed little or no growth at the end of drought.

Figure 6.7 shows plant growth rate (PGR) during early drought compared with growth rate during the recovery period. The 4 genotypes (i.e. 219, 193, 186 and 133) that grew most rapidly during early drought also regrew most rapidly after drought. Conversely, plants with lower than mean growth rates at early drought did not recover very well. The plants with intermediate growth rates under early drought varied considerably in their growth rate during recovery.

Figure 6.8 shows that the plant growth rate [$100 \times \log_{10} (x + 1)$] did not increase after irrigation for most ACD plants and for both *L. multiflorum* and *F. arundinacea* parent species during the recovery period as compared to herbage production during the last 18 days of the drought period. However the growth rate of some ACD plants (Nos. 219, 193, 186, 133, 167, 81 and 33) was higher in recovery after irrigation. Although the growth of *L. multiflorum* was lower than *F. arundinacea* before the end of drought, after irrigation , growth and recovery of *L. multiflorum* was greater than for the *Festuca* species.

In summary, Figures 6.6, 6.7 and 6.8 demonstrate that a few ACD plants i.e. Nos. 219, 193, 133 186 and 167 had high growth rate under drought conditions but were inferior to the pentaploid hybrid which was particularly drought resistant. Growth rates of most ACD plants was lower than either parent species.

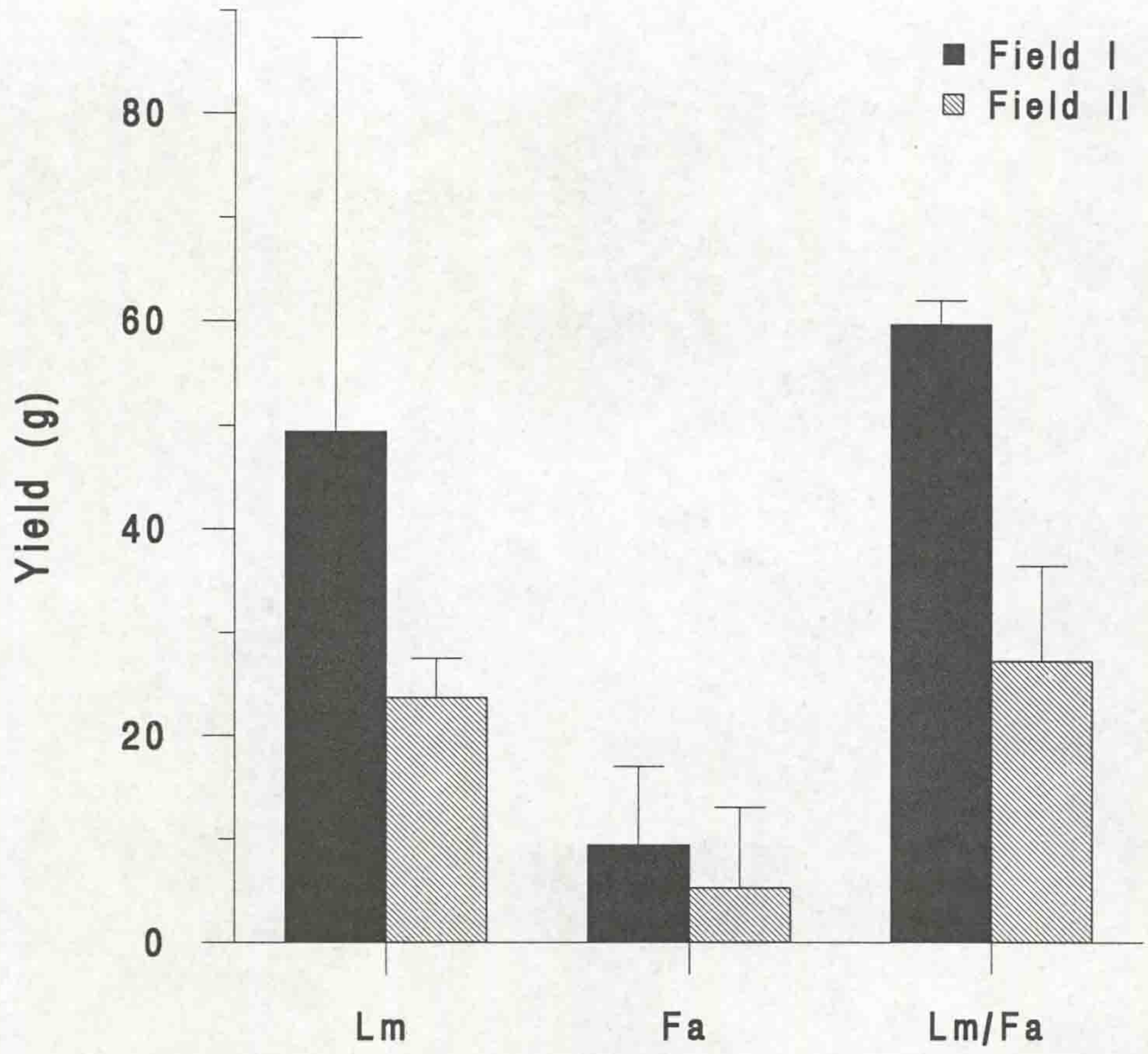


Figure 6.2. Dry herbage matter of control genotypes at Cut 1, prior to onset of drought in the field drought experiment. Field I was nominated for the drought environment and field II as the irrigated control environment. Lm = *L. multiflorum*, Fa = *F. arundinacea*, Lm/Fa = *Lm/Fa* pentaploid hybrid.

Figure 6.3. Yield of dry matter at the second cut, after 92 days of drought (yield of 87 days), using yield of Cut 1 (prior to onset of drought) as covariate. Each point is the mean of two replicates. E1 = Droughted environment, E2 = Irrigated environment, *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea*, *L/F* = *L. multiflorum* × *F. arundinacea* pentaploid hybrid and M = mean of ACD plants.

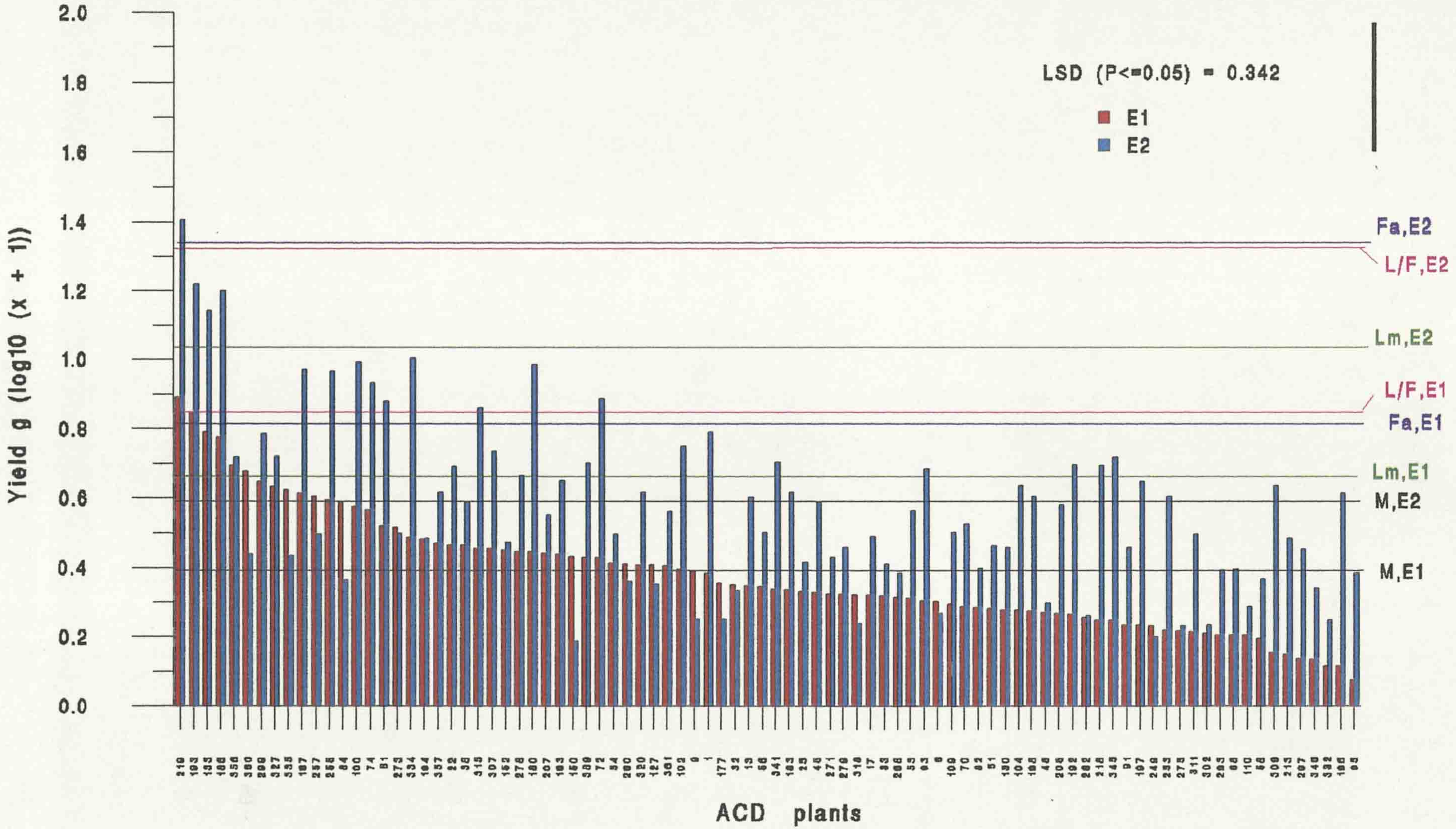

LSD (P<=0.05) = 0.342
E1
E2
Fa,E2
L/F,E2
Lm,E2
L/F,E1
Fa,E1
Lm,E1
M,E2
M,E1
Yield g (log10 (x + 1))
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ACD plants

Figure 6.4. Yield of dry matter at the third cut, after 110 days of drought (growth over 18 days), using yield of Cut 1 (prior to onset of drought) as covariate. Each point is the mean of two replicates. E1 = Droughted environment, E2 = Irrigated environment, *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea*, *L/F* = *L. multiflorum* × *F. arundinacea* pentaploid hybrid and M = mean of ACD plants.

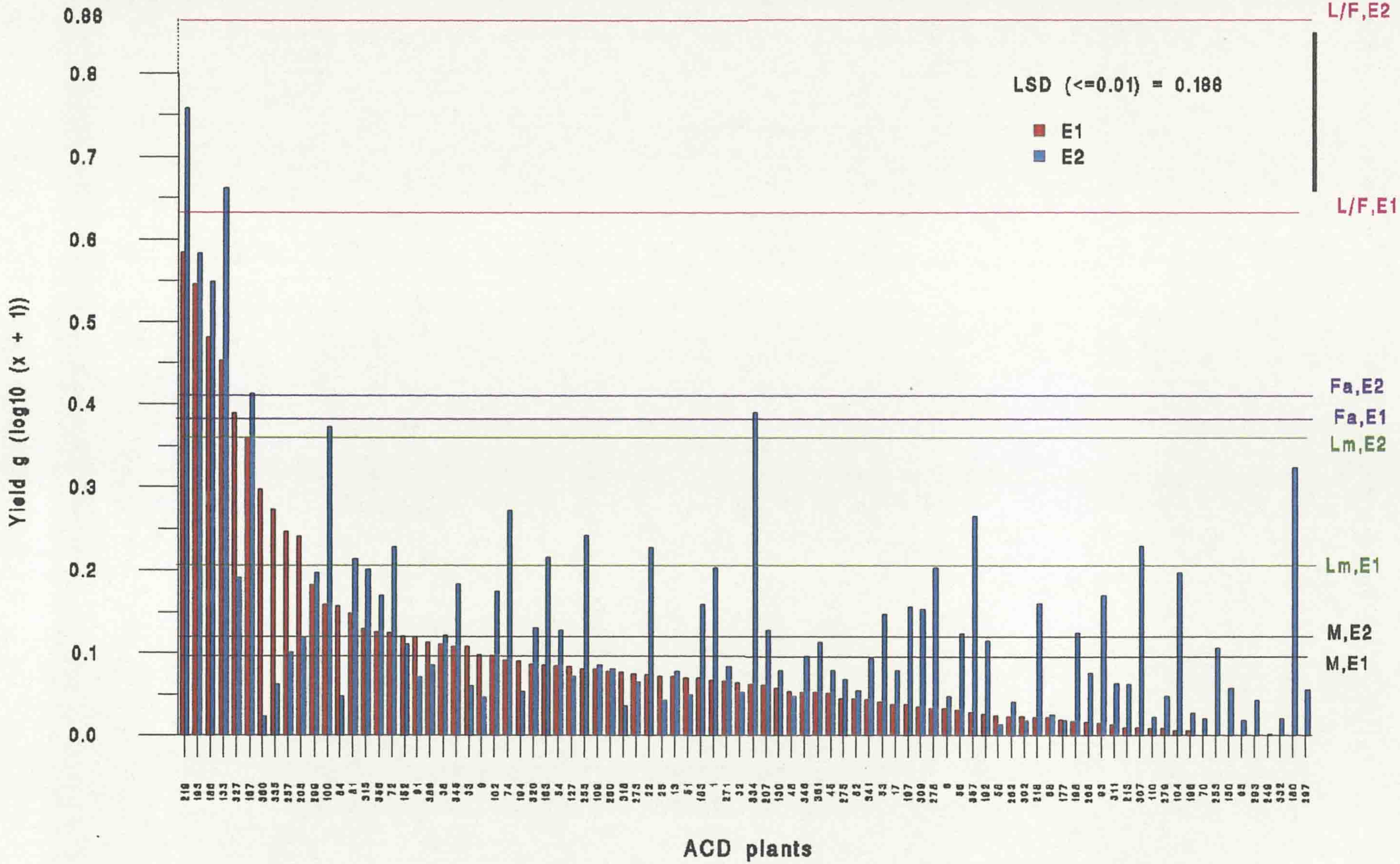
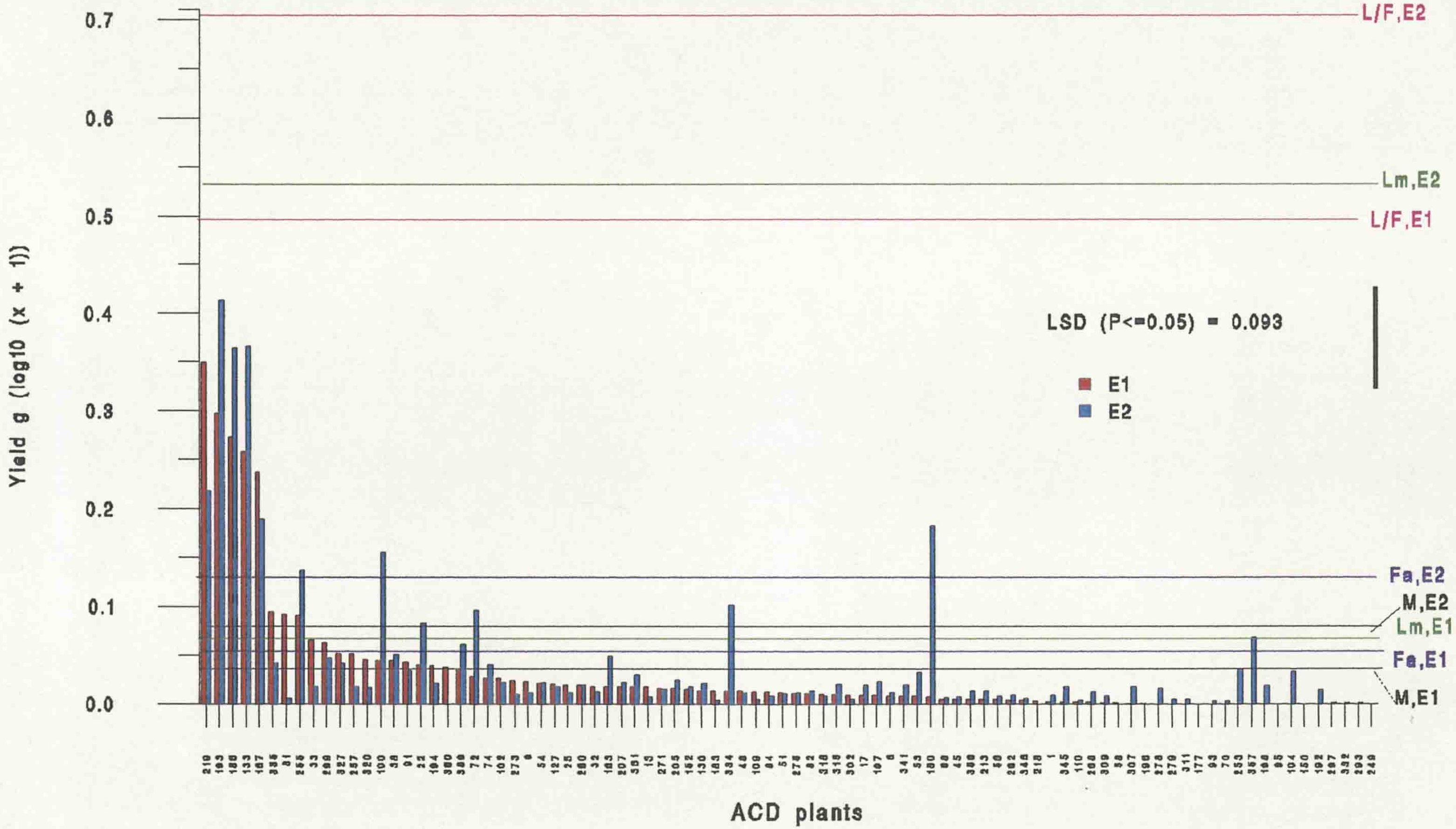

0.88
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Yield g (log10 (x + 1))
LSD (<=0.01) = 0.188
E1
E2
L/F,E2
L/F,E1
Fa,E2
Fa,E1
Lm,E2
Lm,E1
M,E2
M,E1
ACD plants

Figure 6.5. Yield of dry matter at the fourth cut, after 44 days recovery following 110 days of drought, using yield of Cut 1 (prior to onset of drought) as covariate. Each point is the mean of two replicates. E1 = Droughted environment, E2 = Irrigated environment, *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea*, *L/F* = *L. multiflorum* × *F. arundinacea* pentaploid hybrid and M = mean of ACD plants.


Yield g (log10 (x + 1))
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
L/F,E2
Lm,E2
L/F,E1
LSD (P<=0.05) = 0.093
E1
E2
Fa,E2
M,E2
Lm,E1
Fa,E1
M,E1
ACD plants

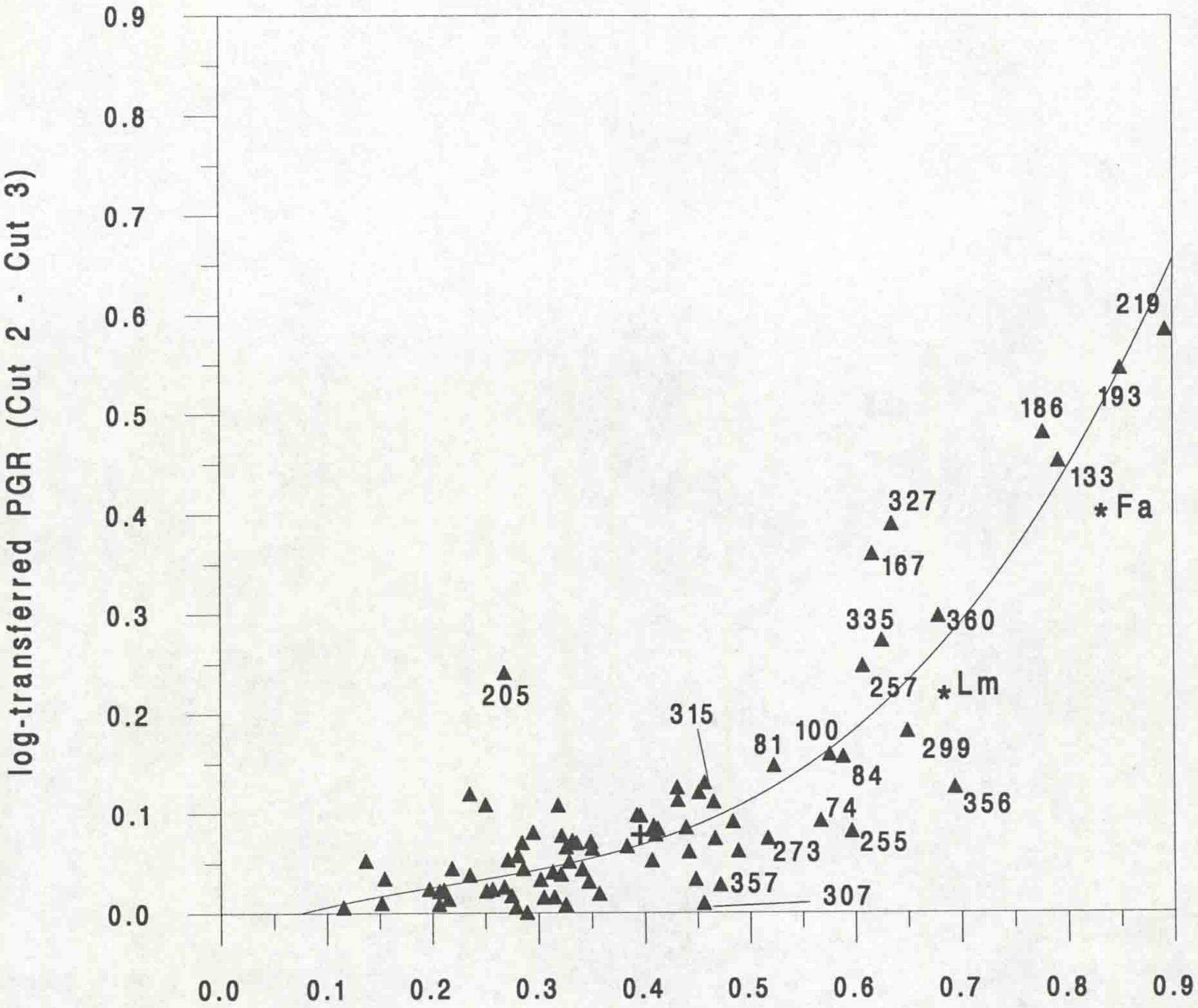


Figure 6.6. Relationship between log-transformed plant growth rate [$100 \times \log_{10}(x + 1)$] after 92 days of drought (yield of 87 days) and at the third cut, after 110 days of drought (growth over 18 days at the end of drought period), using yield of Cut 1 (prior to onset of drought) as covariate. Each point is the mean of two replicates. *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea*, *L/F* = *L. multiflorum* × *F. arundinacea* pentaploid hybrid, and + = mean of ACD plants. Selected genotypes are labelled.

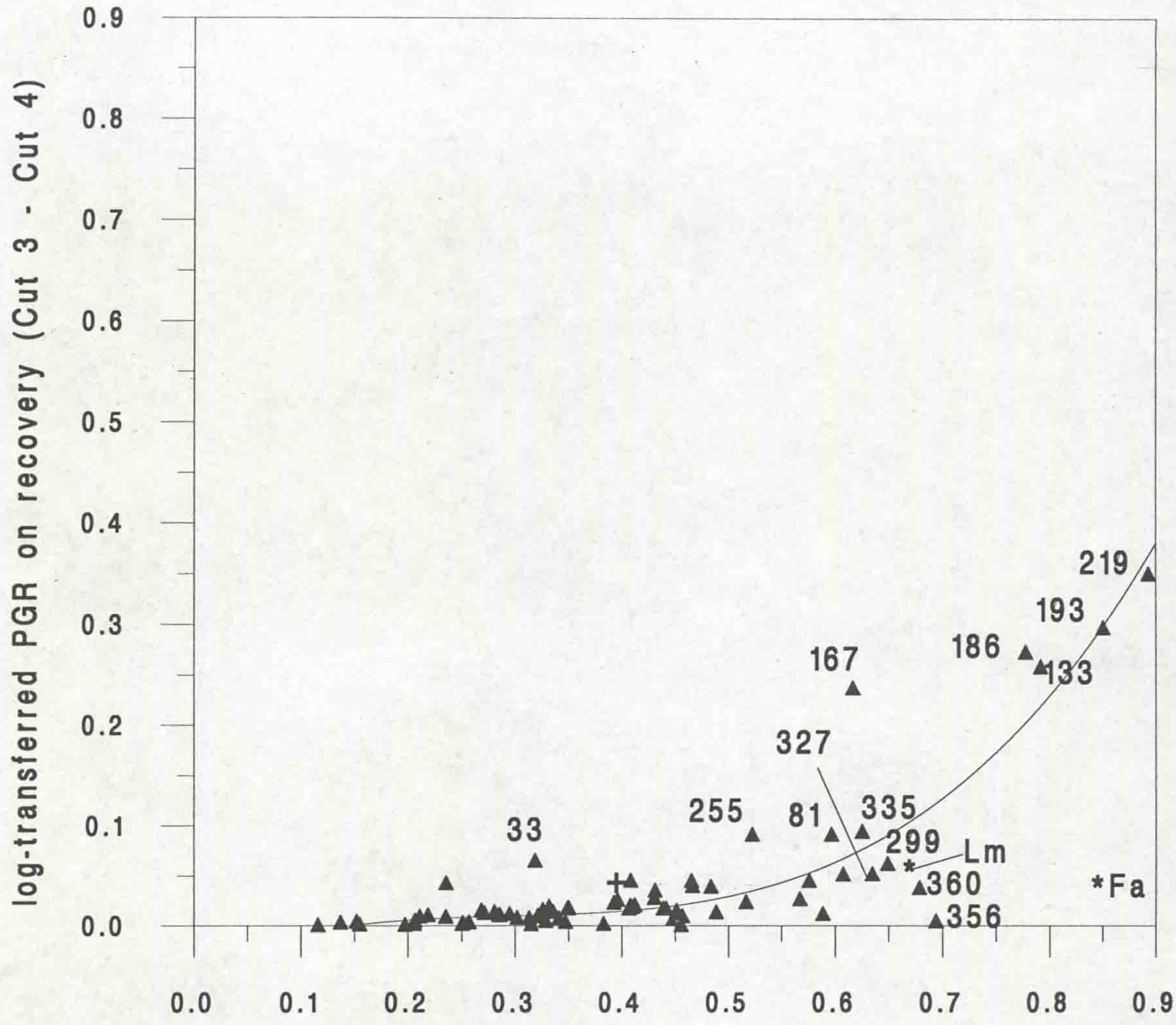


Figure 6.7. Relationship between log-transformed plant growth rate [$100 \times \log_{10}$ (x + 1)] after 92 days of drought (yield of 87 days) and the fourth cut, after 44 days recovery following 110 days of drought and, using yield of Cut 1 (prior to onset of drought) as covariate. Each point is the mean of two replicates. *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea*, *L/F* = *L. multiflorum* × *F. arundinacea* pentaploid hybrid, and + = mean of ACD plants. Selected genotypes are labelled.

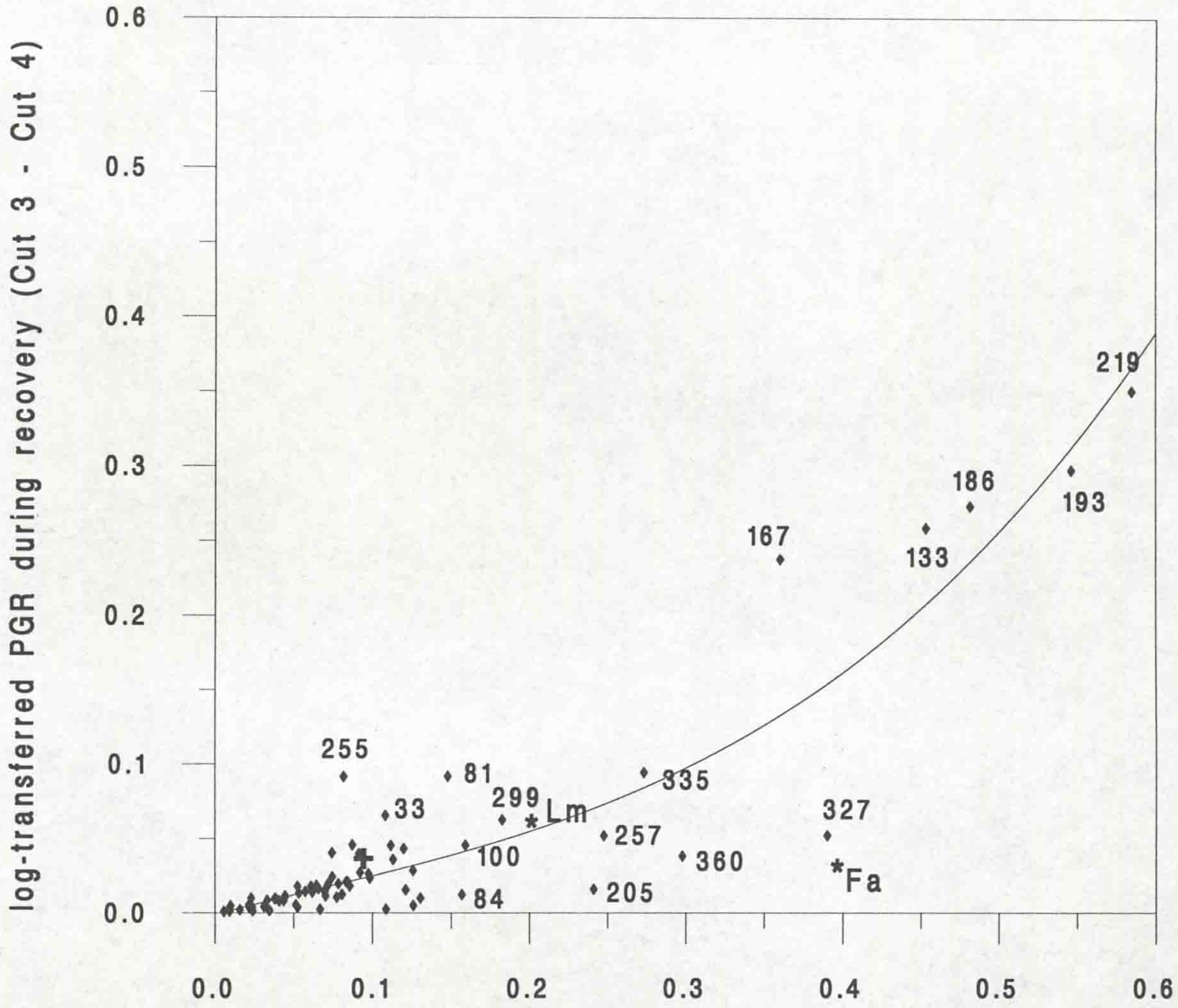


Figure 6.8. Relationship between log-transformed plant growth rate [$100 \times \log_{10}$ (x + 1)] at the third cut, after 110 days of drought (growth of 18 days at the end of drought period) and fourth cut, after 44 days recovery following 110 days of drought and, using yield of Cut 1 (prior to onset of drought) as covariate. Each point is the mean of two replicates. *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea*, *L/F* = *L. multiflorum* × *F. arundinacea* pentaploid hybrid, and + = mean of ACD plants. Selected genotypes are labelled.

6.4.1.3. Tiller production

Normally, germination will not occur without sufficient water being available. Effects of water deficiency normally occur during the early stages of development, during the main growth stages and later on during the kernel filling period. A decrease of tillering frequency commonly follows from water deficiency at the early stages of plant development (Winkel, 1989). Since forage grasses are either grazed or cut for hay, tiller production (as one component of forage yield) is an important factor in breeding forage grasses.

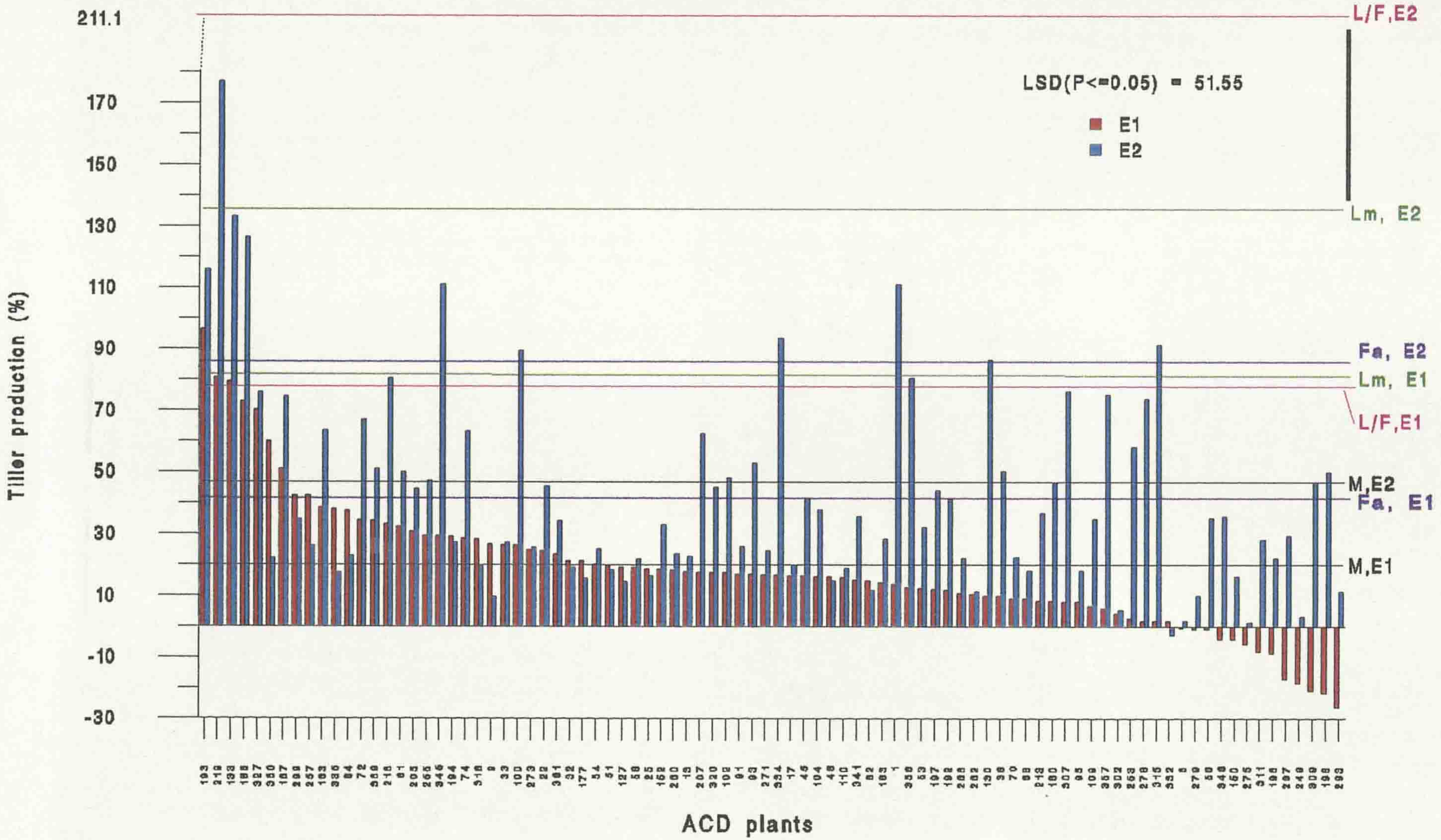
Plants respond to drought stress in many different ways. Cessation in growth commonly occurs in plants under severe water stress and is advantageous to plant survival. Under less severe stress such as that described here, a total cessation of growth would not be an acceptable character to fulfil the demands for growth of forage grasses under temperate growing conditions. Levels of tiller production may be considered as one indicator of a plant's vigour and resistance to drought. Tall fescue normally produces less tillers than Italian ryegrass and this might contribute to its greater tolerance to water stress since it has less demand for water. However, maintaining tiller production of Italian ryegrass in combination with the stress tolerance of the fescue would be the ideal goal for plant breeders.

Analysis of variance of tiller production (the ratio of tiller number at the end of drought (T2) to tiller number at the beginning of drought (T1) over 110 days (between Cut 0 and Cut 3) of drought, is presented in Appendix 6.5. Both genotype and drought treatment significantly influenced the tiller production index ($P \leq 0.001$). Figure 6.9 shows that in the droughted environment only ACD plant 193 exceeded the tiller production found for *L. multiflorum*. This genotype produced significantly more tillers than *F. arundinacea* ($P \leq 0.05$). There were 6 more ACD plants (Nos. 219, 133, 186, 327, 360 and 167) which had a slightly higher tiller production than *F. arundinacea*. In the irrigated environment only plant 219 produced more tillers than Italian ryegrass and was significantly ($P \leq 0.01$) higher

tillering than tall fescue. Eight other ACD plants (Nos. 193, 133, 186, 345, 100, 334, 1 and 315) produced slightly more tillers than tall fescue.

Tiller production in the irrigated environment (46.2) was double that in the droughted environment (20.5); the differences were highly significant ($P \leq 0.01$). The genotype × environment interaction was of border-line significance ($P = 0.0634$). Most ACD plants and all control genotypes produced more tillers in the irrigated environment than in the droughted environment. Seventeen plants, Nos. 219, 133, 186, 345, 100, 334, 1, 356, 130, 307, 357, 253, 278, 315, 297, 309 and 198 produced significantly more tillers in the irrigated environment than in the droughted environment. A few ACD plants produced more tillers in the droughted compared with the irrigated environment e.g. Nos. 360, 257, 335 and 9. In 13 ACD plants e.g. Nos. 293, 198 and 309, tiller number decreased during the drought treatment. Conversely, with the exception of genotype 332, tiller production amongst all ACD plants and control genotypes increased under the irrigated regime. Tiller production was, as expected, higher for Italian ryegrass than tall fescue, a difference that in the irrigated environment was statistically ($P \leq 0.05$) significant. Differences in tiller production between the irrigated and droughted environments were not significant for *Festuca*, but *Lolium* ($P \leq 0.05$) and the pentaploid hybrid ($P \leq 0.01$) produced more tillers in the irrigated environment. The tiller production of *L. multiflorum* was not significantly more than that of *F. arundinacea* in the droughted treatment, but was significantly greater in the irrigated environment. In the droughted plot, the pentaploid hybrid produced almost the same number of tillers as *L. multiflorum.* In the irrigated plot, the pentaploid produced substantially more ($P \leq 0.01$) tillers than both parent species.

Figure 6.9. Tiller production index (tiller number at Cut 3 after 110 days of drought over number of tillers at Cut 0 (start of drought experiment)). Each point is the mean of two replicates. E1 = Droughted environment, E2 = Irrigated environment, *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea* and *L/F* = *L. multiflorum* × *F. arundinacea* pentaploid hybrid.


211.1
170
150
130
110
90
70
50
30
10
-10
-30
Tiller production (%)
LSD(P<=0.05) = 51.55
E1
E2
L/F,E2
Lm, E2
Fa, E2
Lm, E1
L/F,E1
M,E2
Fa, E1
M,E1
ACD plants

The relationship between tiller production index and dry herbage mass of ACD plants at Cut 2 (growth over 87 days) after 92 days of drought, Cut 3 at the end of 111 days of drought (growth over 18 days) and Cut 4 (growth over 44 days in recovery following drought) in the droughted plot is shown in Figures 6.10, 6.11 and 6.12. There were ACD plants with similar tillering number which demonstrated difference in production of dry herbage mass e.g. genotypes 95 and 356 (Figure 6.10) or 257 and 167 (Figure 6.12). Some genotypes even with a negative tiller production index e.g. Nos. 198, 293, 309 and 249 produced more herbage mass during drought than that produced by other genotypes with increasing numbers of tillers during the drought period e.g. No. 95 (Figure 6.10). The high yielding genotypes in all three cuts e.g. Nos. 219, 193, 186 and 133 had the highest tiller production index. Certain genotypes with a large tiller production index had small, narrow, or rolled leaves and thus did not produce large amounts of dry herbage e.g. genotypes Nos. 327 and 360 in the recovery cut (Figure 6.12).

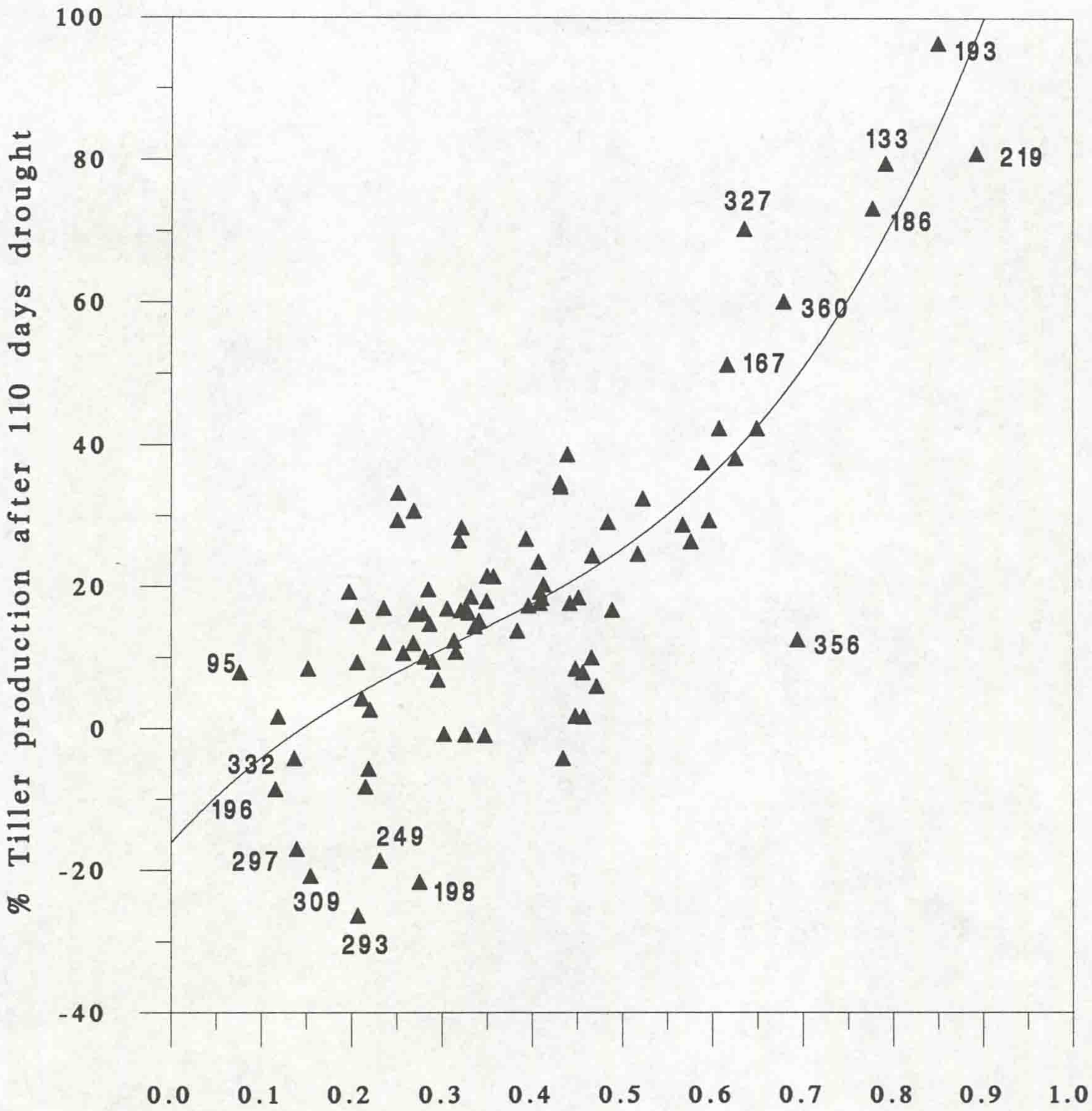


Figure 6.10. Relationship between log-transformed herbage mass per plant after 92 days of drought (growth over 87 days) at Cut 2 and tiller production index at the end of the drought (Cut 3) after 110 days. Selected genotypes are labelled.

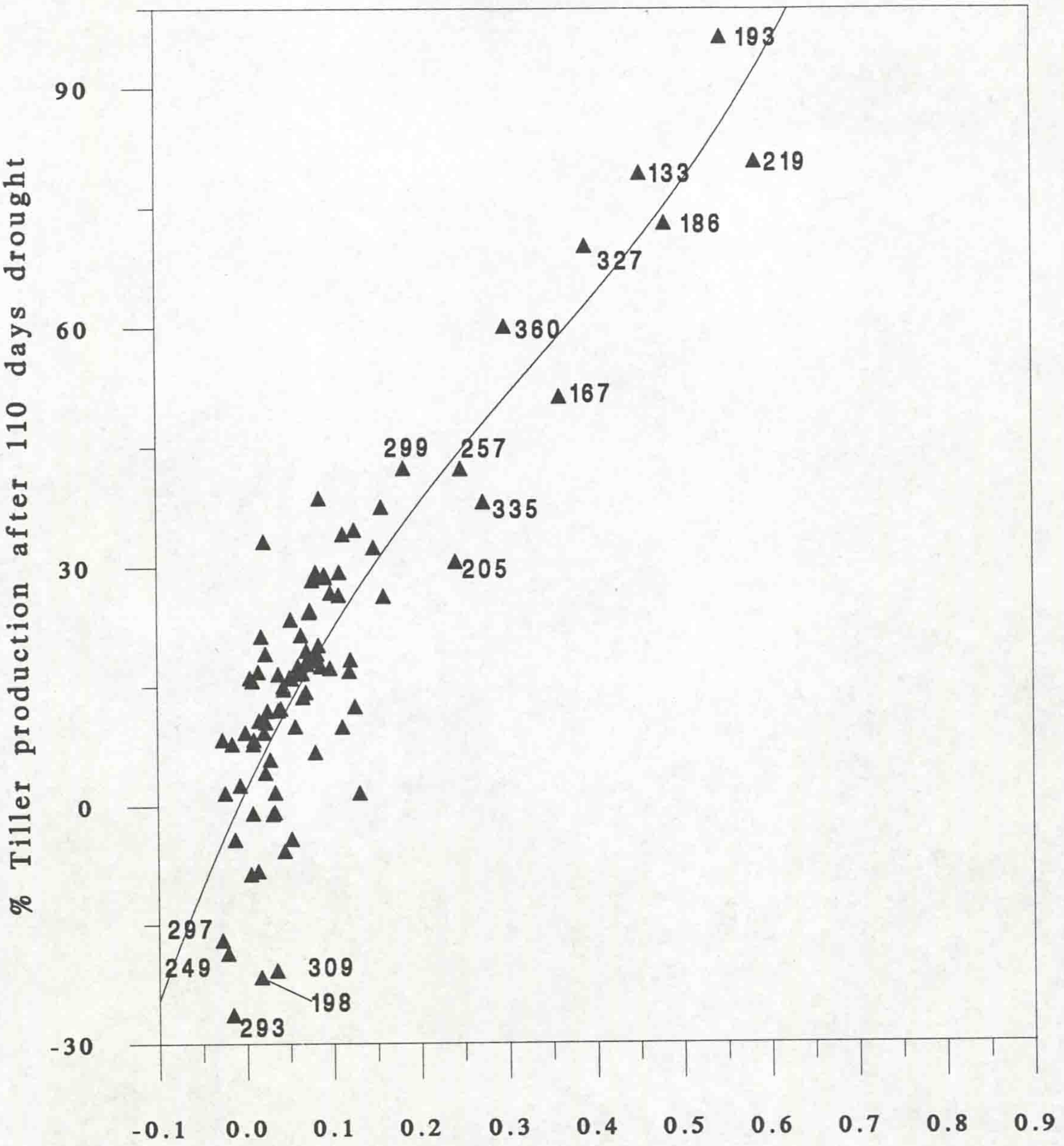


Figure 6.11. Relationship between log-transformed herbage mass per plant after 110 days of drought (growth over 18 days) at Cut 3 and tiller production index at the end of the drought (Cut 3) after 110 days. Selected genotypes are labelled.

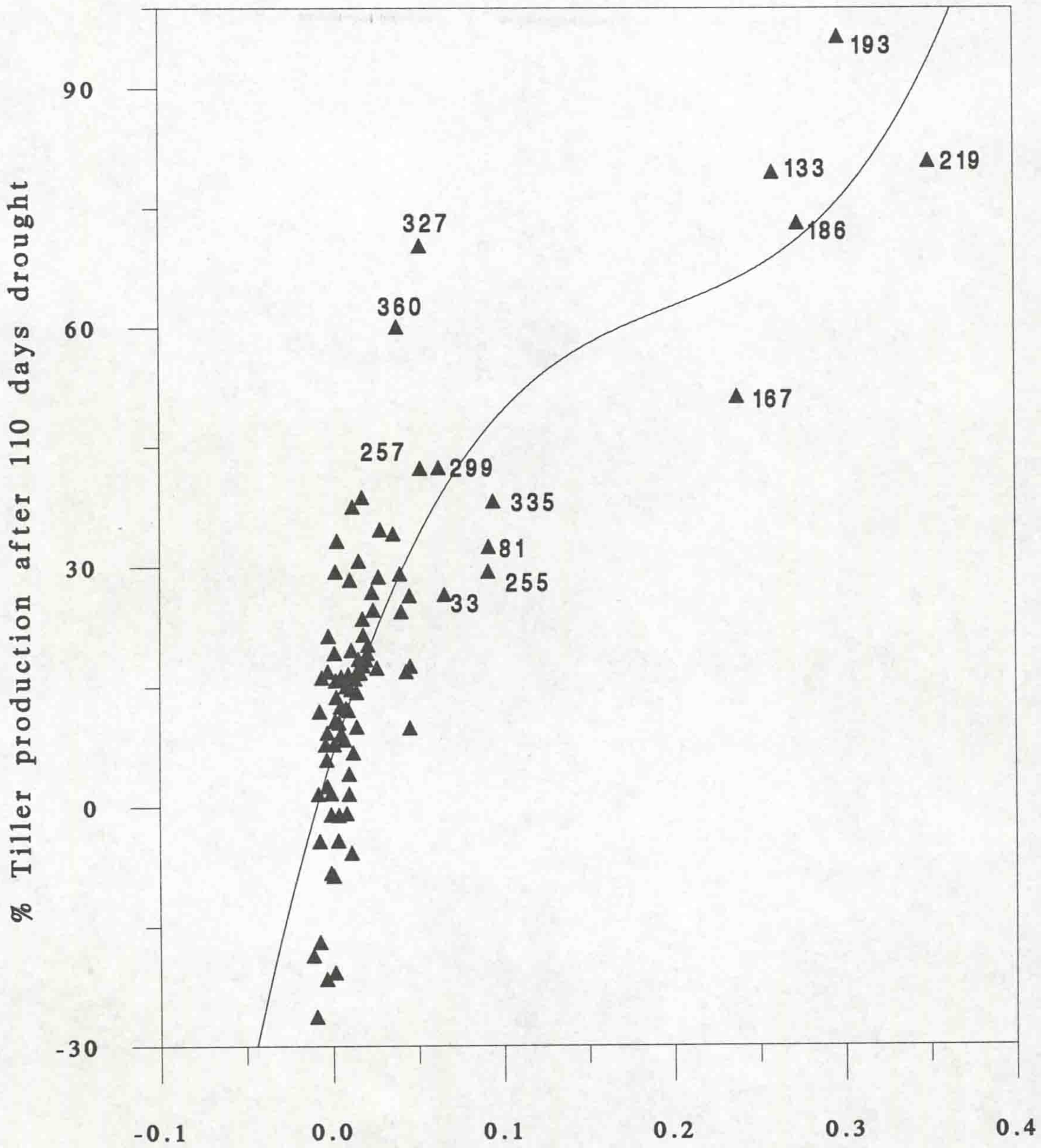


Figure 6.12. Relationship between log-transformed herbage mass per plant at Cut 4, after 44 days recovery following 110 days of drought and tiller production index at the end of drought (Cut 3) after 110 days. Selected genotypes are labelled.

6.4.2. Glasshouse drought experimental results

A second drought experiment was established in 1995 in a glasshouse to minimise the interference of some variables identified previously between drought and irrigated replications in field trials. In particular, effort was made to reduce the error in determining survival under drought because of differences in plant size.

The drought tolerance of each ACD plant was determined by comparing genotype survival following a period of drought where each genotype commenced the drought experiment as a single tiller, as previously carried out in the freezing test experiment. This provided uniformity among the plants and reduced error due to differences in plant size, growth rate, and vigour. Although the temperature of the glasshouse was considered uniform throughout, there were minor climatic differences possibly affecting growth conditions between bins located on the North or South sides of the glasshouse. In order to determine the extent of variation between the North and South sides, replications were analysed in two blocks (5 replications as North block and 5 replications as South block).

Analysis of variance of survival of ACD plants and control genotypes at the end of drought experiment (after 77 days drought) is presented on Appendix 6.6. Although sunshine direction is an important factor on plant growth, the position of blocks on the North and South side of the glasshouse, with survival score means of 4.33 and 4.45 respectively, had no obvious affect on plant survival during drought. As expected, irrigation strongly ($P \leq 0.0001$) influenced levels of plant survival. Differences between genotypes in their survival following drought were highly significant ($P \leq 0.0001$). Also the interaction of genotypes $\times$ irrigation was very significant ($P \leq 0.0001$). In other words, it is possible to detect genotypes that showed a wide range of drought resistance.

The range of variation between ACD plants in survival after drought is illustrated in Figure 6.13. Only ACD plants Nos. 193, 2', 22, 346 and 100 survived drought better than *F. arundinacea*, and a further 16 ACD plants (Nos. 193, 2', 22,

346, 100, 56, 219, 167, 188, 315, 279, 293, 64, 152, 345 and 218) withstood drought better than *L. multiflorum*. Tolerance to drought was significantly higher ($P \leq 0.05$) for ACD plant 193 than for *F. arundinacea,* and for ACD plants Nos. 193, 2' and 22 was significantly higher than for *L. multiflorum*. More than half of the ACD plants (61.7%) were significantly less drought tolerant than the drought sensitive control species, *L. multiflorum*. Twenty one ACD plants died as a result of drought.

Survival of ACD plants was very different in droughted and irrigated conditions. Unexpectedly, some genotypes demonstrated significantly better survival under drought than under daily irrigation e.g. Nos. 193, 22, 219, 167, 74, 341, 334, 102, 35, 72, 186, 255, 79 and 182. In some cases, ACD plants which survived under drought, died in the irrigated bins e.g. 22, 167, 341, 334, 102 and 35.

Other genotypes, as expected, performed significantly better under irrigated conditions compared with the drought treatment. e.g. 293, 345, 218, 258, 253, 400, 51, 84, 9, 413, 375, 312, 1, 311, 6 and 178. A total of 13 plants which survived under irrigated conditions died under drought e.g. 192, 38 and 501. Eight ACD plants (Nos. 494, 308, 273, 272, 243, 215, 52 and 33) died under both irrigated and droughted conditions. Some ACD plants had more or less the same survival condition in both environments e.g. 2', 100, 279, 64, 152, 359, 257, 78 and 39.

L. multiflorum control plants and guard plants especially in the irrigated bins displayed rapid growth during the drought treatment. The high growth rate of *L. multiflorum* indicates its value as a forage grass. It is likely that Italian ryegrass utilises most of its assimilates in shoot production rather than by developing its root system (Garwood *et al.* 1979). Thus in drought conditions, loss of water presumably increases in *L. multiflorum* because of increases in the ratio of above ground biomass compared to root growth. Slow root growth will limit access to moisture from deeper layers of soil. Stress from drought could have been exacerbated by flowering but because of frequent cutting, the effect of flowering on survival under drought was probably negligible, and plants seldom changed from vegetative to reproductive phase.

Although initially *Lolium* had a higher growth rate than *Festuca*, by the end of the drought treatment, the fescue survived better than ryegrass under both drought and irrigated conditions. The pentaploid hybrid had intermediate drought tolerance between the two parent species but in the irrigated controls, its growth and survival was inferior to either parent species.

Analysis of variance indicating levels of recovery following 33 days irrigation is described in Appendix 6.7. There was no effect on recovery of bin position in the glasshouse (North or South facing bins had survival score means of 4.02 and 4.06 respectively). Recovery between genotypes was significantly different ($P \leq 0.0001$). Although the effect of environment was not significant, the genotype $\times$ environment interaction was highly significant ($P \leq 0.0001$).

Variation between ACD plants in recovery with irrigation following drought was considerable (see Figure 6.14). The overall mean scores of droughted and irrigated treatments were similar (4.07, and 3.90 respectively), and replicates did not differ statistically. On the other hand, recovery between individual genotypes was different in the previously droughted bins and the controls. A total of 14 ACD plants (Nos. 491, 488, 460, 457, 414, 361, 321, 307. 398, 375, 210, 121 and 113), which survived the drought, died during the recovery period both in the droughted bins and also in the irrigated controls. Seven ACD plants (Nos. 501, 411, 410, 335, 268, 246 and 184) which apparently failed to survive the drought, in fact recovered, and produced some green leaves after irrigation. These seven plants appear to demonstrate an alternative form of drought tolerance to the parent species, more characteristic of hot dry climates, by total cessation of growth under drought followed by recovery after the onset of irrigation. Unlike the seven ACD plants, the Fescue parent, despite decreasing growth rate under drought-stress (Figure 6.14), continued to produce leaves and tillers. Further investigation will be necessary to ascertain whether the gene combinations obtained by anther culture in these 7 ACD plants, encode for an alternative survival strategy to that found in the parental species.

ACD plants 346, 193, 2' 22, 413, 100 and 375 showed slightly better recovery than the drought resistant control species (*F. arundinacea*) and a total of 27 ACD plants had superior recovery to *L. multiflorum*, and this was significant ($P \leq 0.05$) for genotypes No. 346, 2', 22, 413 and 375. Twenty nine plants recovered better than the pentaploid hybrid with differences significant in the case of Nos. 346, 2', 22, 413, 100 and 375.

Leaf production of *F. arundinacea* and the pentaploid hybrid plant in recovery following drought was significantly higher than in irrigated clonal control replicates. This emphasises the natural drought tolerance of the fescue which is capable of maintaining sufficient reserves during drought to allow rapid regrowth once access to water returns. The opposite response was observed in the drought-sensitive *L. multiflorum* genotypes which had higher growth rate in the control bins at levels exceeding their clonal counterparts when in recovery following droughting.

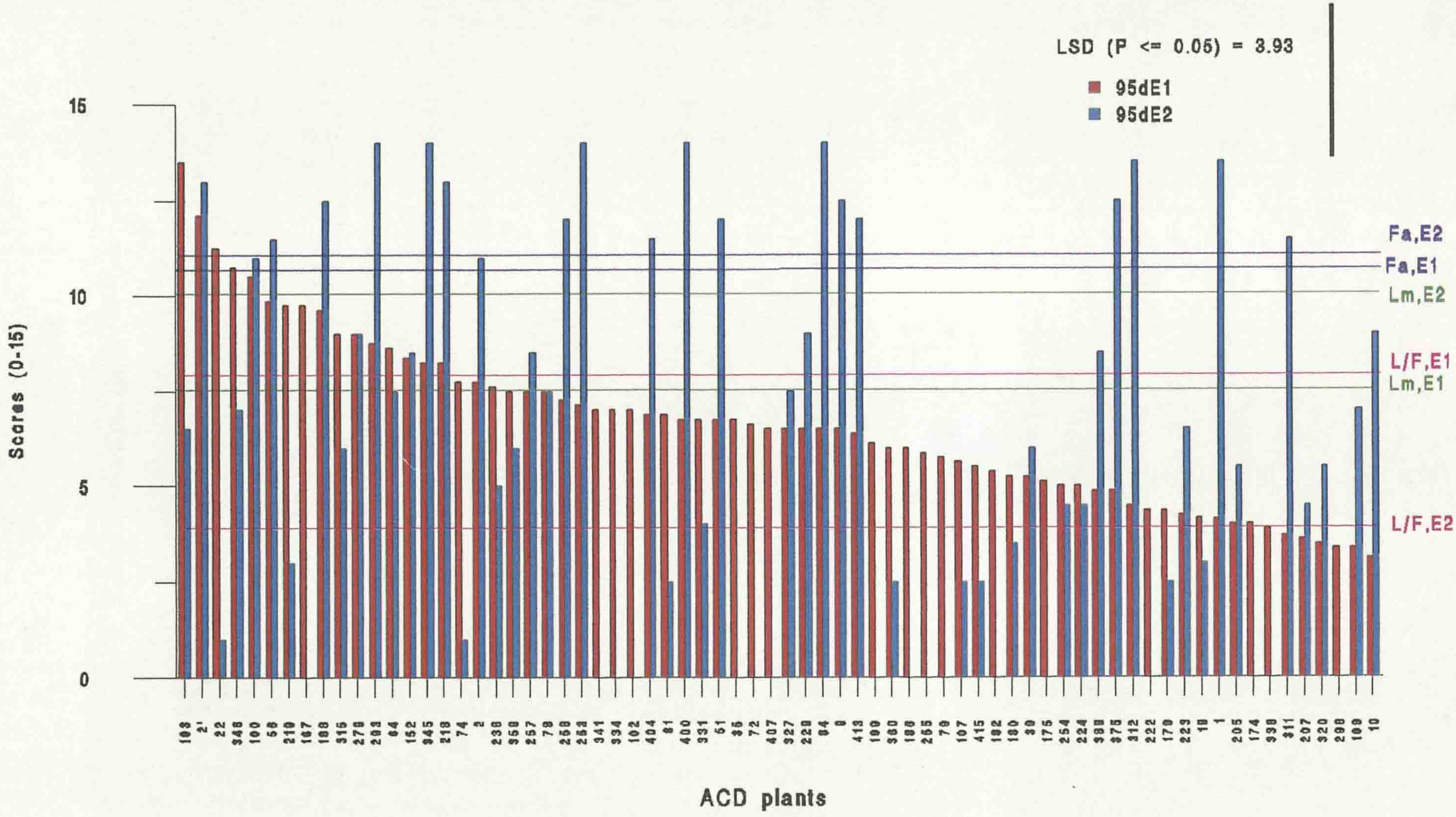
The response of individual ACD plants to the return of regular watering varied between genotypes. Some ACD plants performed better than *F. arundinacea* i.e. 346, 193 and 2' and better than the pentaploid hybrid e.g. 22, 413, 100, 375, 400 and 229. Other genotypes, despite recovering well following drought, were unable to maintain growth rates in the irrigated controls e.g. 22, 413, 81, 255 and 79. Other ACD plants behaved similarly to *L. multiflorum* with better performance in recovery under regular irrigation e.g. 293, 9, 258, 2, 1, 341, 175, 336 and 183. Other ACD plants showed no indication of any detrimental effect due to previous water-stress, producing as much foliage in recovery after drought as their respective control replications e.g. 346, 56, 188, 219 and 360.

At the end of the drought period, a total of 35 ACD plants died in the control bins compared with only 21 ACD plants in the droughted replicates. During the drought period in summer 1995, the temperature inside the glasshouse was very high. The effect of the high temperature was an additional stress factor in this experiment. Differences in growth under drought and regular irrigation need to take into account the additional effect of heat-stress. Acclimation to drought conditions

might enhance levels of resistance to heat shock. This is in agreement with the results of Volaire and Thomas (pers. comm.) who found that plants of *L. perenne* and *Dactylis glomerata* which were acclimated to severe drought were much more tolerant to simulated heat shock than were well-irrigated plants.

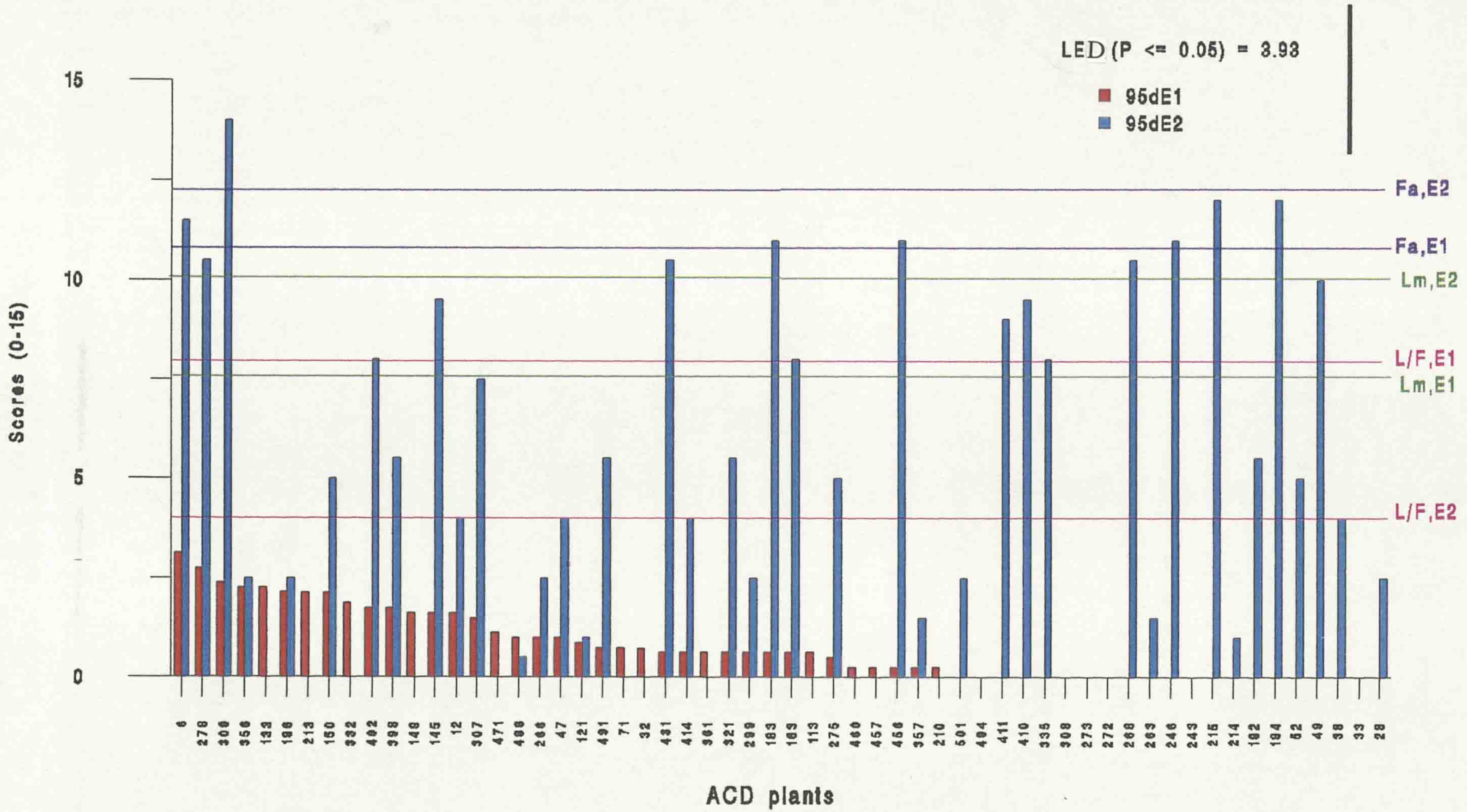
Some of the most drought resistant ACD plants demonstrated significantly better survival under drought than under daily irrigation; indeed, some of the control replicates died in the irrigated bins. These drought resistant plants e.g. Nos. 193 and 219 had high growth rates (Chapter 4) and also showed high freezing tolerance (Chapter 5). Regular watering would appear to reduce the ability of these genotypes to withstand high temperatures.

Figure 6.13. Survival score of ACD plants at the end of drought in a glasshouse, after 77 days drought. Each point is the mean of 8 replications in the droughted replicates and 2 replicates in the irrigated replicates. No. 1 to 501 = ACD plants, Lm = *L. multiflorum*, Fa = *F. arundinacea* and L/F = *L. multiflorum* × *F. arundinacea* pentaploid hybrid.


LSD (P <= 0.05) = 3.93
95dE1
95dE2
15
10
5
0
Scores (0-15)
Fa,E2
Fa,E1
Lm,E2
L/F,E1
Lm,E1
L/F,E2
103 21 22 348 100 58 210 167 188 315 278 293 64 152 345 218 74 2 238 358 257 79 258 253 341 334 102 404 81 400 331 61 35 72 407 327 220 84 0 413 190 380 188 255 79 107 415 192 180 30 175 254 224 388 375 312 222 170 223 18 1 205 174 338 311 207 320 298 109 10
ACD plants

160a.

Figure 6.13 (continued). Survival score of ACD plants at the end of drought in a glasshouse, after 77 days exposed to drought. Each point is the mean of 8 replicates in the droughted replicates and 2 replicates in the irrigated replications. No. 1 to 501 = ACD plants, Lm = *L. multiflorum*, Fa = *F. arundinacea* and L/F = *L. multiflorum* × *F. arundinacea* pentaploid hybrid.


LED (P <= 0.05) = 3.93
95dE1
95dE2
Fa,E2
Fa,E1
Lm,E2
L/F,E1
Lm,E1
L/F,E2
Scores (0-15)
15
10
5
0
6
278
306
356
133
196
213
150
332
402
398
148
145
12
307
471
466
266
47
121
491
71
32
431
414
361
321
289
183
163
113
275
460
457
456
357
210
501
404
411
410
335
308
273
272
268
263
246
243
215
214
182
194
52
49
38
33
28
ACD plants

161a

Figure 6.14. Survival score of ACD plants after 33 days recovery following 77 days exposed to drought. Each point is the mean of 8 replicates in the droughted replicates and 2 replicates in the irrigated replicates. No. 1 to 501 = ACD plants, Lm = *L. multiflorum*, Fa = *F. arundinacea* and L/F = *L. multiflorum* × *F. arundinacea* pentaploid hybrid.

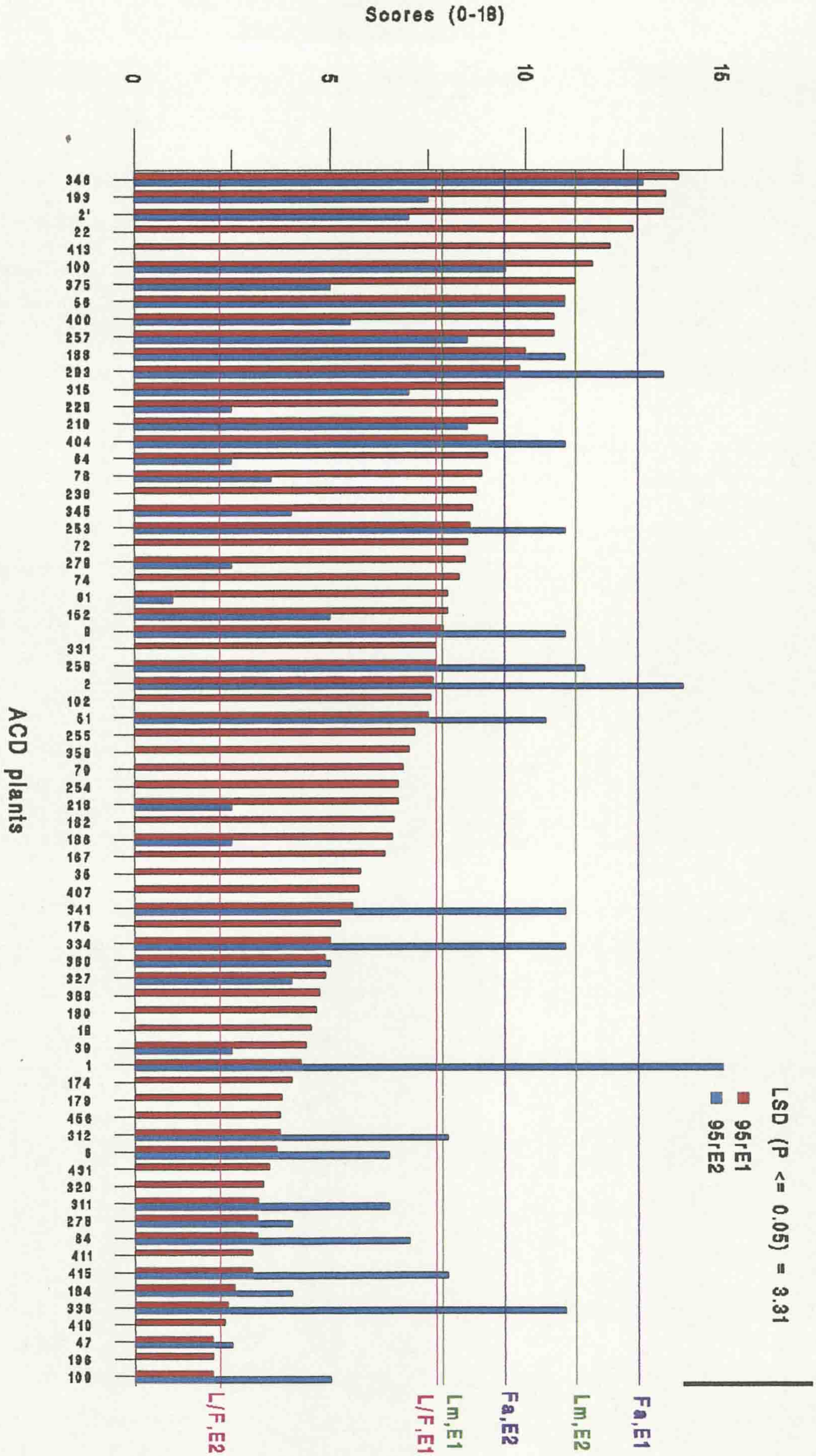
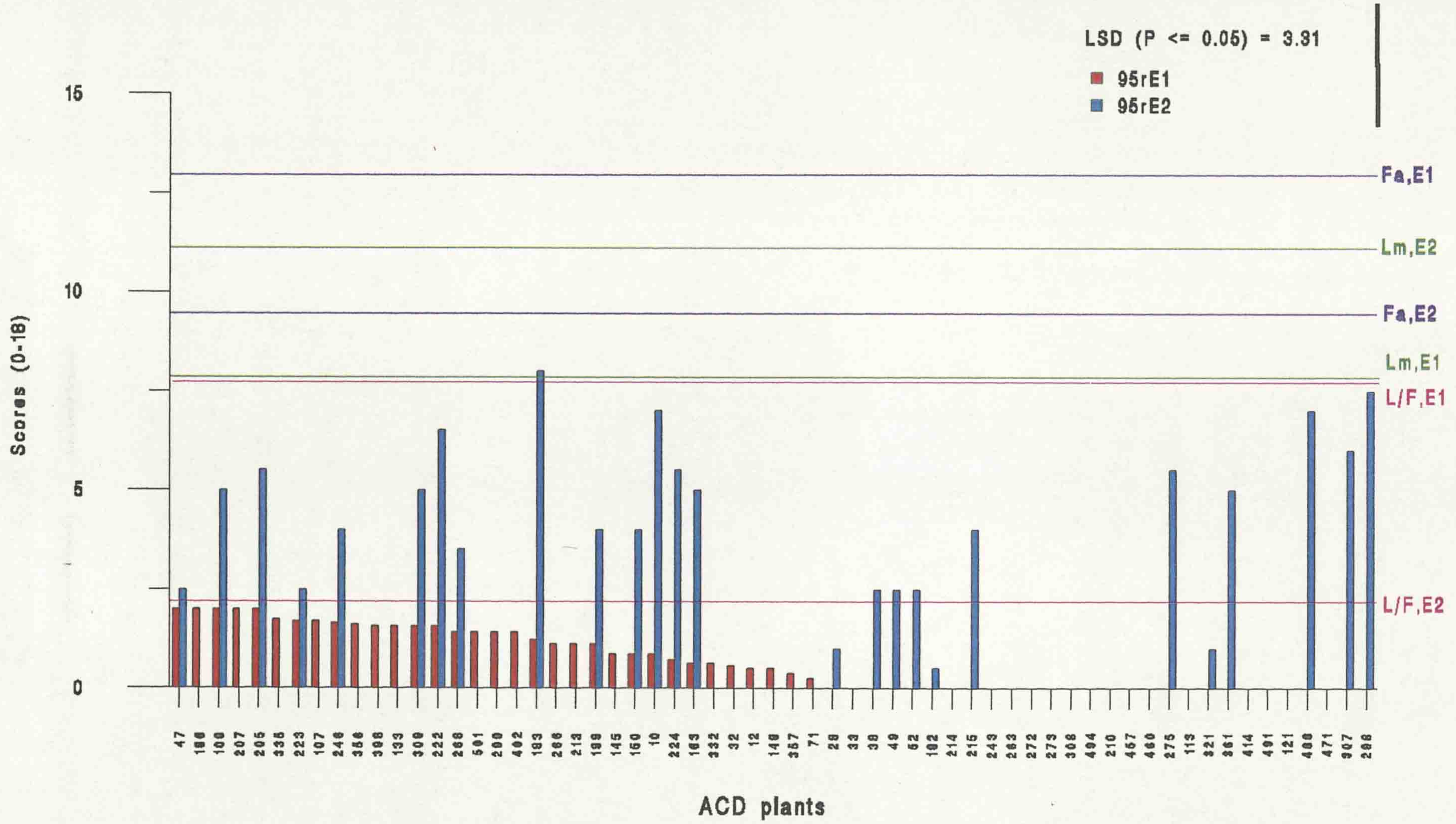

Scores (0-18)
0
5
10
15
ACD plants
346
199
2'
22
413
100
375
56
400
257
188
293
315
228
210
404
64
78
238
345
253
72
278
74
61
152
8
331
258
2
102
51
255
358
79
254
218
182
186
167
36
407
341
175
334
360
327
389
180
18
39
1
174
179
456
312
6
431
320
311
278
84
411
415
184
336
410
47
196
109
LSD (P <= 0.05) = 3.31
95rE1
95rE2
Fa,E1
Lm,E2
Fa,E2
Lm,E1
L/F,E1
L/F,E2

Figure 6.14 (continued). Survival score of ACD plants after 33 days recovery following 77 days exposed to drought. Each point is the mean of 8 replicates in the droughted replicates and 2 replicates in the irrigated replicates. No. 1 to 501 = ACD plants, Lm = *L. multiflorum*, Fa = *F. arundinacea* and L/F = *L. multiflorum* × *F. arundinacea* pentaploid hybrid.


LSD (P <= 0.05) = 3.31
95rE1
95rE2
Fa,E1
Lm,E2
Fa,E2
Lm,E1
L/F,E1
L/F,E2
15
10
5
0
Scores (0-18)
47 156 100 207 205 335 223 107 246 356 306 133 300 222 268 501 200 402 183 266 213 199 145 150 10 224 163 332 32 12 148 357 71 28 33 38 49 52 102 214 215 243 263 272 273 308 494 210 457 460 275 113 321 361 414 491 121 488 471 907 298
ACD plants

1636

6.4.3. The relationship between drought resistance under field and glasshouse conditions

The correlation analysis of data from the drought resistance experiments under the field and the glasshouse conditions is presented in Appendix 6.8. The relationship between herbage mass (g/plant) and tiller production index (TPI) (%) in the droughted environment under the polythene rain-out shelter in the field and survival score in the bins under the glasshouse conditions is illustrated in Figures 6.15, 6.16 and 6.17. There was a weak correlation coefficient ($r = 0.42$, Appendix 6.8) between herbage mass during the last 18 days of drought under the field conditions and survival score after 77 days of drought under the glasshouse conditions (Figure 6.15). Although the two most drought resistant ACD plants in the field experiment (219 and 193) had a good survival score in the glasshouse experiment, the *Lm/Fa* pentaploid hybrid which consistently produced more herbage than any other genotype under drought in the field, did not maintain this high ranking under glasshouse conditions. Some genotypes which showed poor growth under drought in the field, such as ACD plant Nos. 22, 56 and 100 had higher survival scores in the glasshouse experiment. Difference in drought resistance in the two experiments could not be explained by simple differences in plant morphology. The ACD plants with different response to drought-stress in the two experiments, (Nos. 22, 56 and 100) showed wide variation in leaf width (No. 56 very narrow, No. 22 median and No. 100 with broad leaf). Difference in leaf shape cannot be the reason for the poor relationship between survival in the glasshouse and yield under drought in the field ($r = 0.27$). Among high yielding genotypes in the field, there were plants (e.g. No. 193) with a maximum survival score and contrastingly also plants (e.g. No. 133) with a low survival score in the glasshouse. Conversely among low yielding genotypes in the field, there were plants with a high survival score in the glasshouse (e.g. No. 22 and *F. arundinacea*) and also many others with zero score of survival, Figure 6.16.

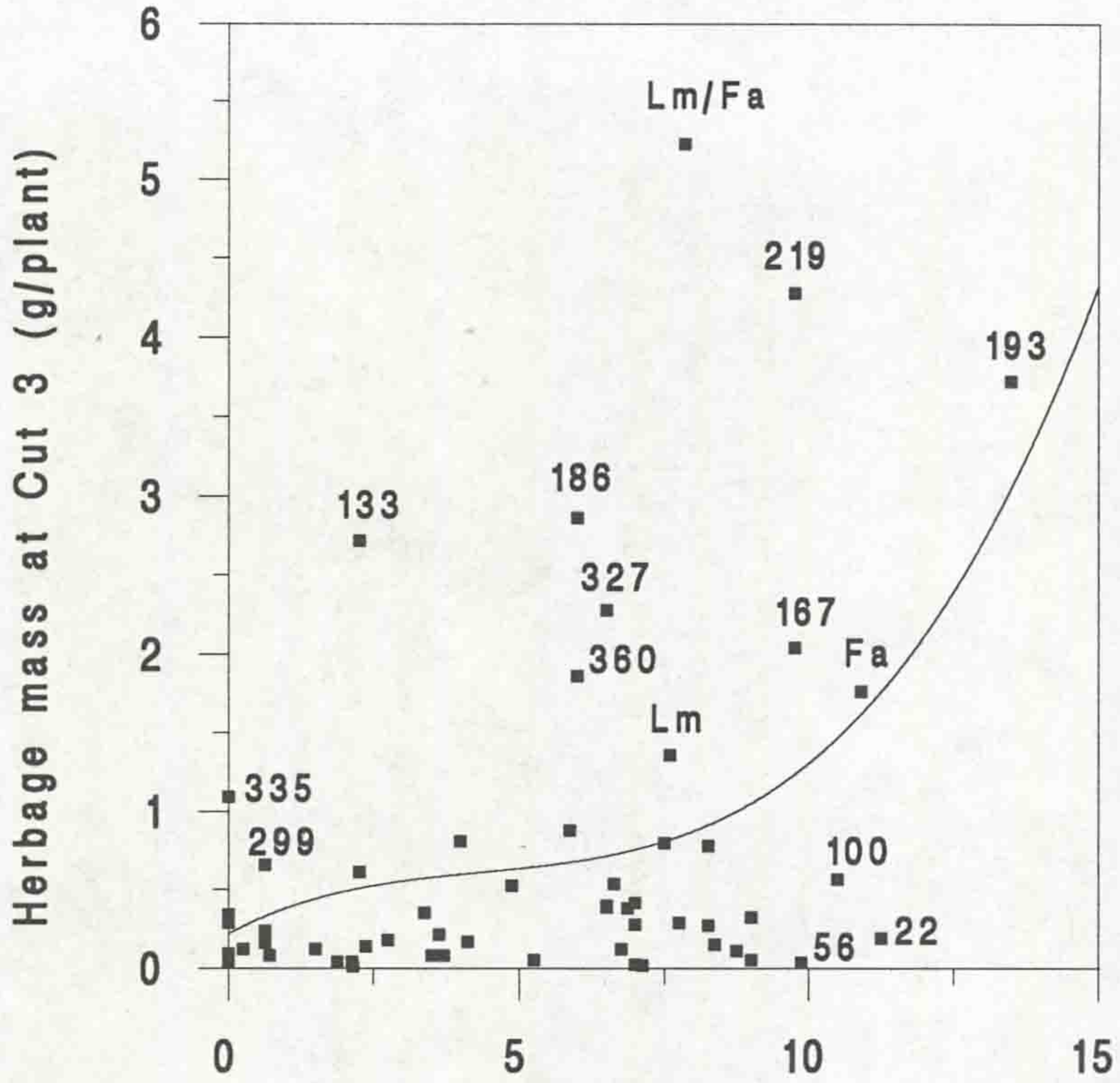


Figure 6.15. Relationship of herbage mass at Cut 3 during the last 18 days of drought in the field drought experiment and survival score (1-15) at the end of 77 days drought under glasshouse conditions of ACD plants and the three control genotypes. Selected genotypes are labelled. Nos. (1-389) = ACD plants number, *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea* and *Lm/Fa* = Pentaploid hybrid.

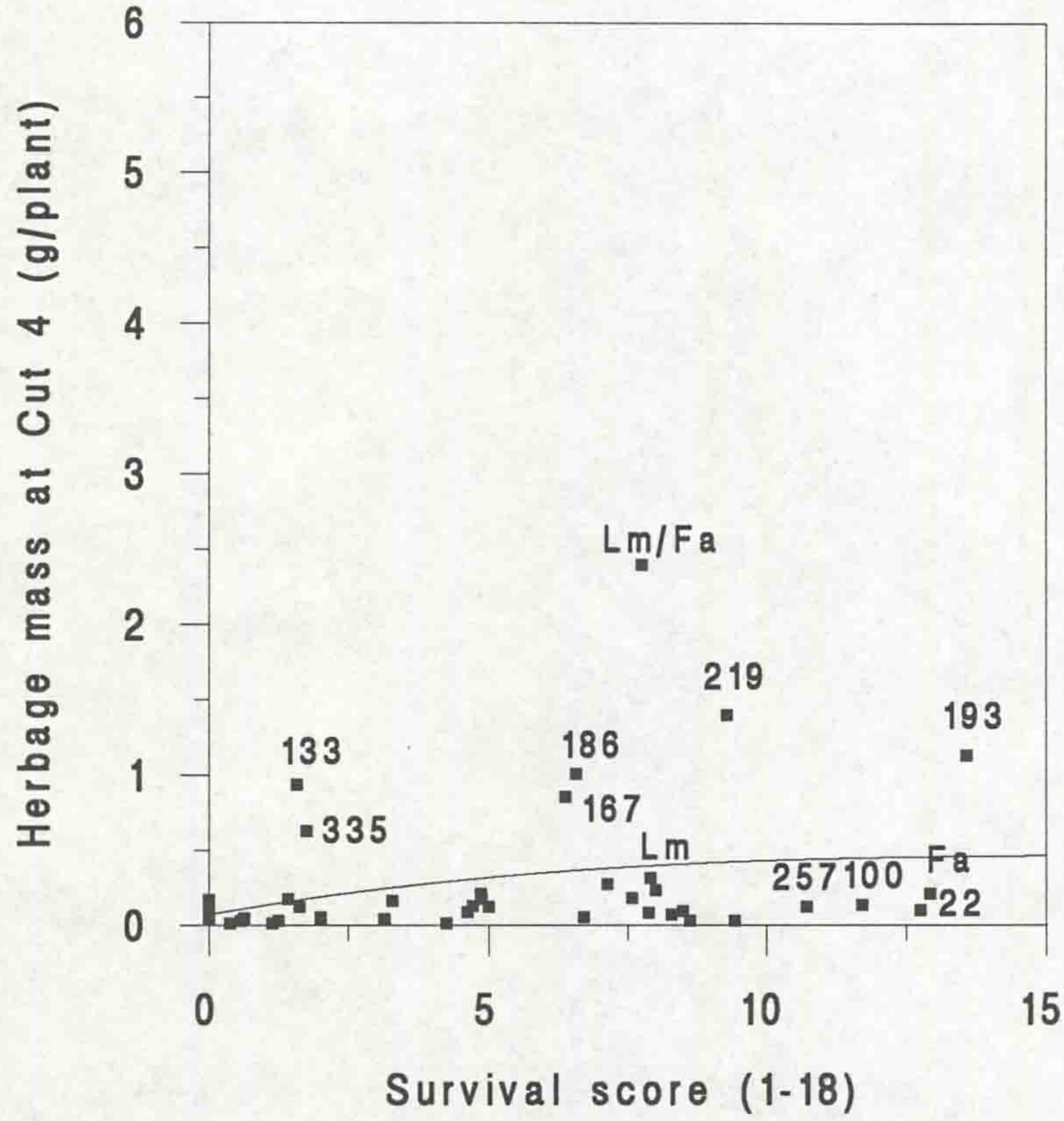


Figure 6.16. Relationship of herbage mass at Cut 4 during 47 days recovery following 110 days drought in the field drought experiment with the survival score after 44 days recovery following 77 days drought under glasshouse conditions of ACD plants and the three control genotypes. Selected genotypes are labelled. Nos. (1-389) = ACD plants number, *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea* and *Lm/Fa* = Pentaploid hybrid.

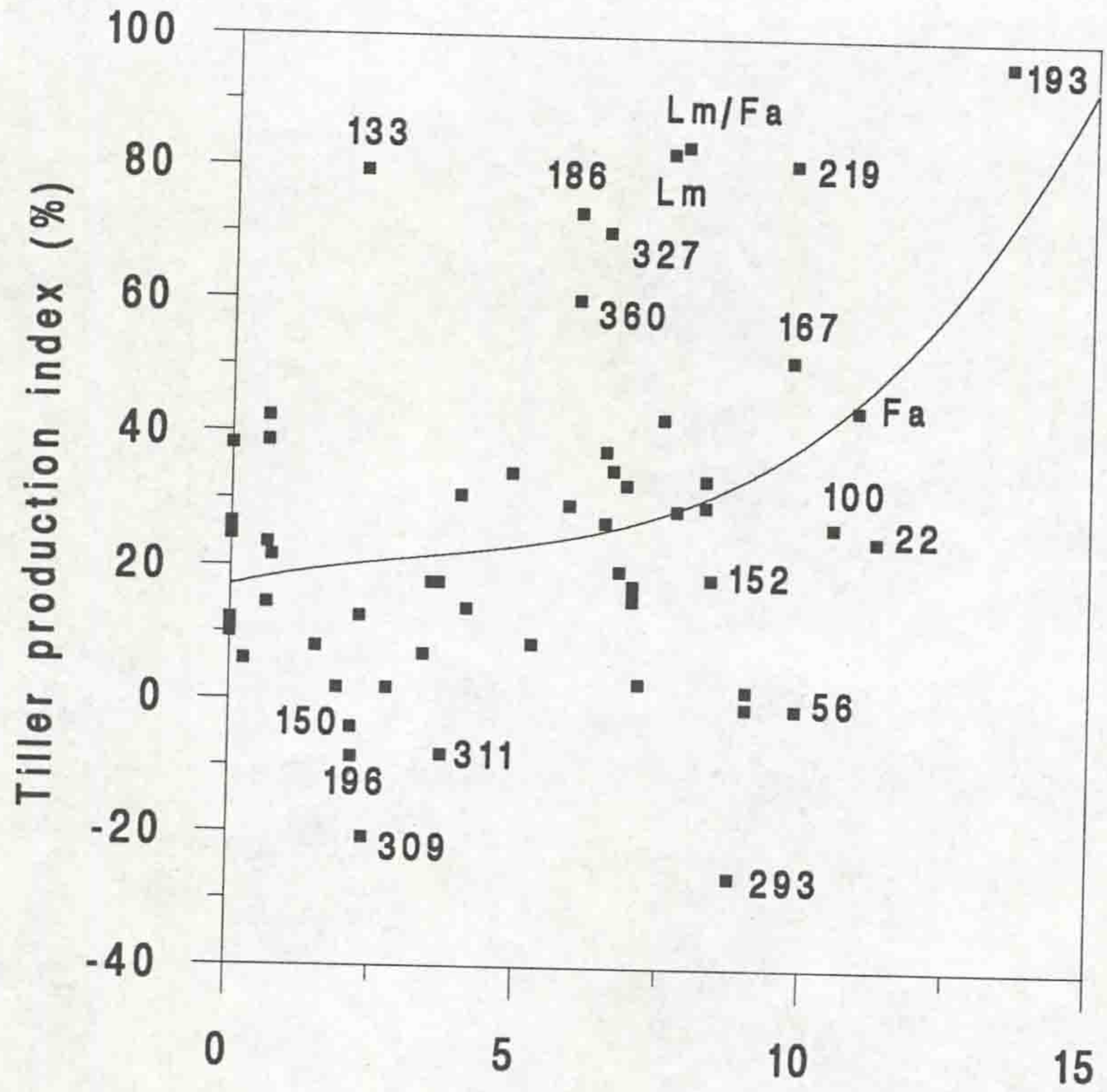


Figure 6.17. Relationship of tiller production index (TPI) in the field during 110 days of the drought experiment (Cut 3) with survival score (1-15) at the end of 77 days drought under glasshouse conditions of ACD plants and the three control genotypes. Selected genotypes are labelled. Nos. (1-389) = ACD plants number, *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea* and *Lm/Fa* = Pentaploid hybrid.

The number of tillers were not correlated with the survival score. The correlation between tiller production index (%) and survival score at the end of the drought experiments was poor (r = 0.41). Figure 6.17 shows that the relationship between these two variables was similar to that between herbage mass and survival score (Figure 6.15).

The plants in the glasshouse faced additional stresses not experienced by plants in the field. These were: a) frequent cutting b) high temperature. During hot days the glasshouse temperature could reach above 40°C with little air movement. The frequent cutting removed the mutual shading and furthermore this stress was higher in plants with the higher growth rate. c) In addition, the soil texture and nutrition value of field was not optimised to the levels in the bins of the glasshouse.

Androgenic genotypes have a mixed assortment of *L. multiflorum*, *F. pratensis* and *F. glaucescens* chromosomes (see chapter 8). Drought resistance is considered a quantitative and complex character (Lazic-Jancic *et al.*, 1995; Marmiroli *et al.*, 1995; Quarrie *et al.*, 1995 and Thomas *et al.*, 1995). Different combinations of genes for drought resistance in ACD plants may have conferred resistance to drought stress and heat-shock in the two drought experiments. Only by conferring complete uniformity under controlled environmental conditions, will it be possible to replicate experiments. Even then, differences in the pretreatment or condition of plants prior to droughting is capable of bringing variation into the response of the same plant to drought stress in different experiments conducted at different times.

Observations confirmed that ACD plants responded to drought in a number of ways. Further investigations will be necessary to identify the different mechanisms involved. One possible mechanism of resource allocation under stress was demonstrated in ACD plant 22. This plant, when cut while under drought stress, exuded very sticky sugar-like sap. This plant which was very high tillering, had very soft and short leaves similar to Italian ryegrass and dark green colour like the fescue parent. The genotype demonstrated excellent recovery following drought

and therefore sufficient reserves to increase growth following the end of the drought period. The plant had prostrate tillers which could reduce evaporation from the soil surface. In contrast, the most drought resistant ACD plants (Nos. 193 and 219) had very long and slightly abrasive leaves (like tall fescue) with bright green colour and upright tillers. A prostrate growth habit therefore was not essential to drought tolerance and indeed is not typical of tall fescue (as its name implies). ACD plant Nos. 193 and 219 had very well developed root systems which must increase the capability of these plants to utilise water and nutrients at depth in the soil.

ACD plants such as No. 56 with very soft, fine, and relatively short leaves, (see chapter 4) showed poor growth in drought and recovery under field conditions but had high survival scores in the glasshouse experiment. This result clearly demonstrates the value of commencing the drought resistance experiment with a single tiller and reducing possible plant competition. The ability to survive high temperatures and drought with low growth rates is more typical of grasses adapted for Mediterranean climates where survival is more important than yield (Volaire, 1995).

ACD plant 219, which had the highest dry matter and the second highest tiller production under drought conditions in the field experiment, was ranked seventh at the end of drought in the glasshouse experiment. Observations showed that genotype 219 like tall fescue (Humphreys and Thomas, 1991) had problems in establishment. The lower survival score in 219 in the glasshouse experiment was mainly due to the failure of two ramets to establish. Without these two ramets, the survival score of plant 219 (survival score = 13.0) would be second to plant 193 (survival score = 13.5) under drought conditions.

In many species (Hoffermann and Parsons, 1991), drought resistant genotypes have low growth rates under drought. However, there were some ACD plants (e.g. Nos. 219, 193, 133 and 186) that were high yielding under both irrigated and drought conditions. Such genotypes (particularly Nos. 193 and 219) demonstrate well some of the desirable complementary characteristics of both *L.*

multiflorum and *F. arundinacea*. Cytological investigations are necessary to find out the parental contribution, and are described in Chapter 8.

6.4.4. Conclusions from the drought experiments

Although many of the ACD plants were less drought resistant than both *L. multiflorum* and *F. arundinacea* species in both experiments, there were some genotypes more drought resistant than *F. arundinacea* but not many. About 6% of ACD plants yielded more dry matter under drought stress than *F. arundinacea* (3% were significantly more drought tolerant than *F. arundinacea*). After recovery following drought, 11% of ACD plants out yielded *F. arundinacea* of which 6% produced significantly greater herbage mass ($P \leq 0.05$). Tiller production of one line (No. 193) was more than both parental species and 10% of ACD plants produced as many tillers as both *L. multiflorum* and *F. arundinacea* species. Survival scores of a third of the ACD plants were not statistically ($P \leq 0.05$) different from *F. arundinacea*.

Humphreys and Thomas (1993) demonstrated that 3% of their *L. multiflorum* introgression lines produced from backcrosses between *Lolium* and the *Lm/Fa* pentaploid hybrid were as drought resistant as *F. arundinacea*. Such a backcross programme takes a minimum of three years and will yield germplasm with introgressed *Festuca* genes in the heterozygous state. The possibility through anther culture of rapid production of doubled polyhaploids and to fix genes for drought resistance in the homozygous state and at higher frequencies than is possible through conventional breeding suggests a future role for anther culture in breeding for improved drought resistance. However, before androgenic plant germplasm can be used in variety production, it must be established that genes selected for drought resistance or inadvertently other deleterious gene combinations have not led to reduced forage quality of ACD plants. One major advantage in the backcrossing

programme described by Humphreys and Thomas (1993) is that *Festuca* genes have been introduced into *Lolium* to increase drought resistance without diminishing the quality characteristics of the ryegrass.

Dry herbage mass while being a very good indicator to select drought resistant ACD plants made no allowance for variations in plant morphology. The survival scores used in the glasshouse experiment were therefore more important as an indicator of drought resistance since they took into account differences in size of plants. Tiller production was not as powerful as a selection criterion for drought resistance as were dry herbage mass and survival score. *L. multiflorum* as a drought susceptible species has a higher tillering capacity than *F. arundinacea* which might indicate why there is little relationship between tiller production and drought resistance.

6.4.5. The relationship between drought and cold resistance

The correlation analysis of data from the freezing tolerance experiments (Chapter 5) and drought resistance experiments is presented in Appendix 6.9. Pearson correlation coefficient between LT_{50} and other measured variables were found to be negative in value implying that the lower the LT_{50} the more likely a plant is to be drought resistant.

The relationship between freezing tolerance (LT_{50}, °C), the PGR (plant growth rate mgd^{-1}) and TPI (tiller production index %) in the drought environment under the polythene rain-out shelter is illustrated in Figures 6.18 to 6.21. During 87 days of drought (Figure 6.18) there was a negative correlation coefficient (r = -0.67, Appendix 6.9) between PGR and LT_{50}. Usually, cold-sensitive genotypes (e.g. ACD plants 293 and 253) were low yielding and cold-tolerant genotypes (e.g. ACD plants Nos. 219, 193, 186, *F. arundinacea* and pentaploid hybrid) were high yielding in the field. Certain high yielding genotypes (e.g. plants 133, 356 and *L.*

multiflorum) were not among the best cold tolerant genotypes. Also within the cold sensitive ACD plants there were some genotypes (e.g. Nos. 327, 74 and 389) which produced average yield, whilst some other low yielding genotypes (e.g. Nos. 332 and 346) corresponded with the average LT_{50}.

Correlations were similar between the PGR at Cut 3, (Figure 6.19) and Cut 2 (r = -0.666 and -0.668 respectively, Appendix 6.9) with cold tolerance LT_{50}. Mainly high yielding genotypes (Nos. 219, 193, 186, *F. arundinacea* and pentaploid hybrid) under drought were tolerant to low temperatures and usually the yield of plants under drought decreased in relation to their freezing tolerance. However, with the exception of certain genotypes (e.g. pentaploid hybrid, plant Nos. 133, 327, 389 and 278; Figure 6.20), the strongest correlation (r = -0.75, Appendix 6.9) between the LT_{50} and drought resistance in the field, was with PGR expressed during the 44 days of recovery period following the drought. Cold tolerant genotypes (plant Nos. 219, 193 and 186 and pentaploid hybrid) except *F. arundinacea* showed good recovery after drought.

The correlation coefficient between tiller production index (%) at the end of the drought period in the field, and freezing tolerance (LT_{50}) was r = -0.56 (Appendix 6.9, Figure 6.21).

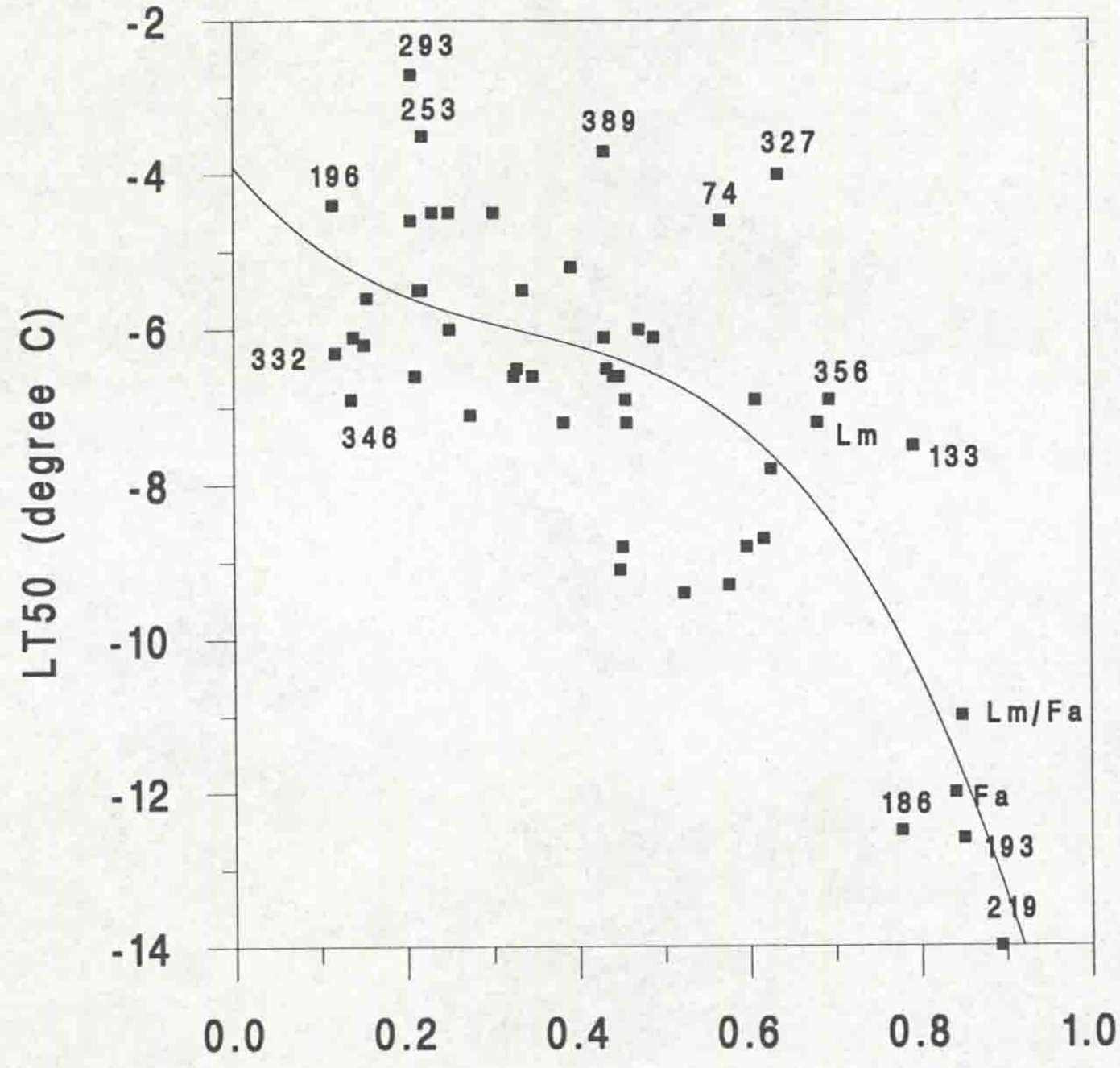


Figure 6.18. Relationship of plant growth rate (PGR) at Cut 2, during 87 days of drought in the field drought experiment with freezing tolerance (LT_{50}) of ACD plants and the three control genotypes. Selected genotypes are labelled. Nos. (1-389) = ACD plants number, *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea* and *Lm/Fa* = Pentaploid hybrid.

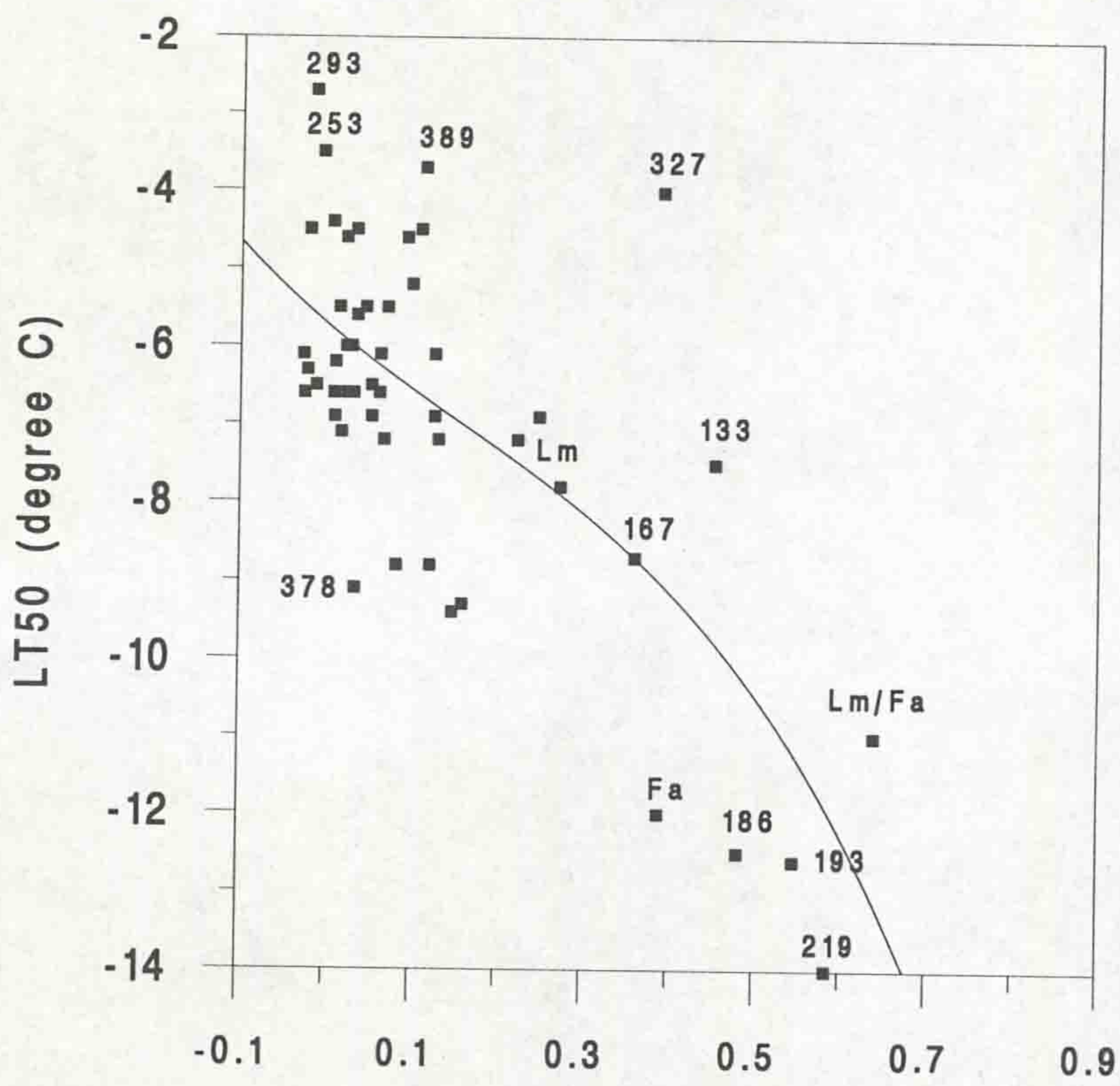


Figure 6.19. Relationship of plant growth rate (PGR) at Cut 3 during the last 18 days of drought in the field drought experiment with freezing tolerance (LT_{50}) of ACD plants and the three control genotypes. Selected genotypes are labelled. Nos. (1-389) = ACD plants number, *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea* and *Lm/Fa* = Pentaploid hybrid.

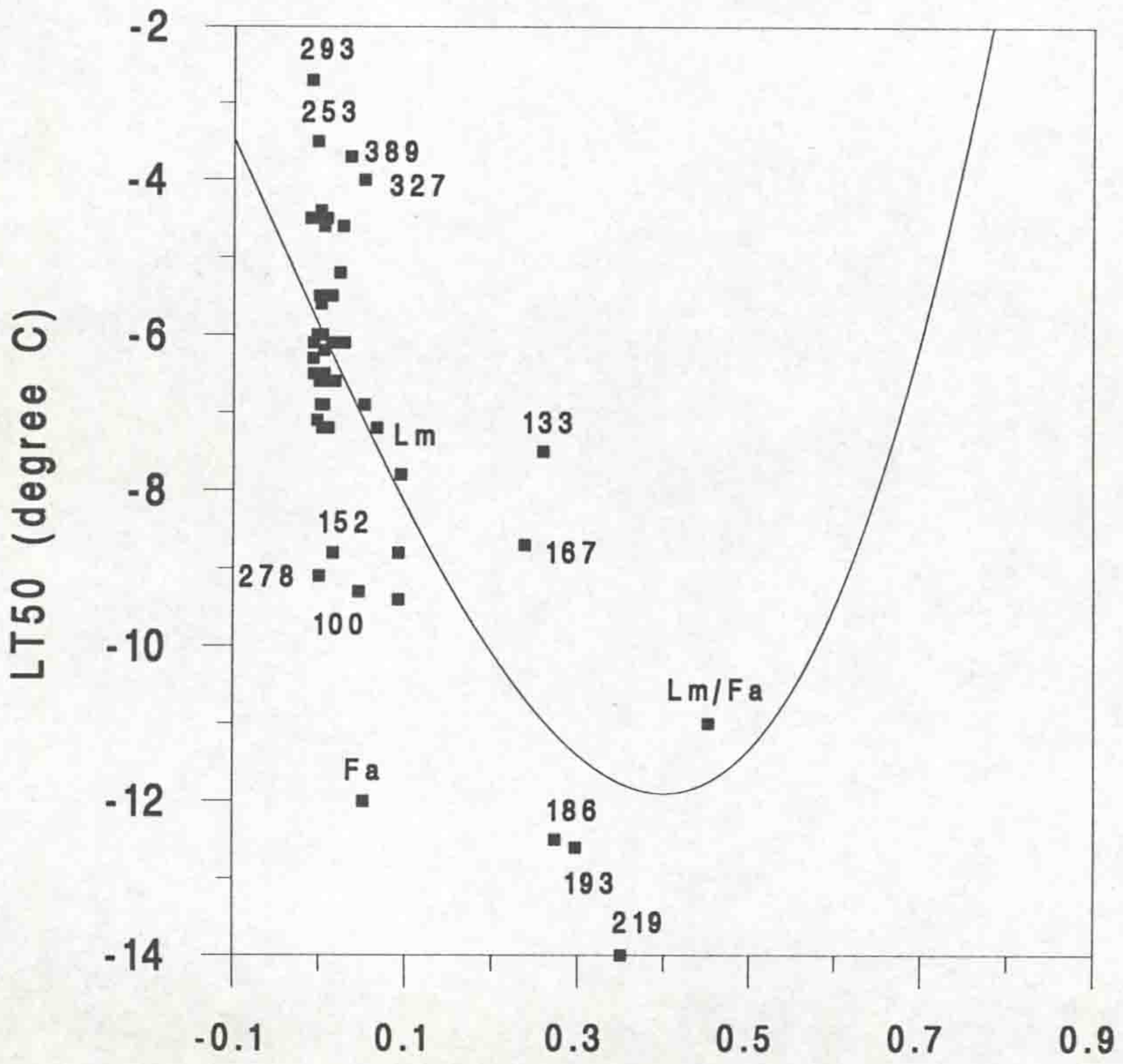


Figure 6.20. Relationship of plant growth rate (PGR) at Cut 4 during 47 days recovery following drought in the field drought experiment with freezing tolerance (LT_{50}) of ACD plants and the three control genotypes. Selected genotypes are labelled. Nos. (1-389) = ACD plants number, *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea* and *Lm/Fa* = Pentaploid hybrid.

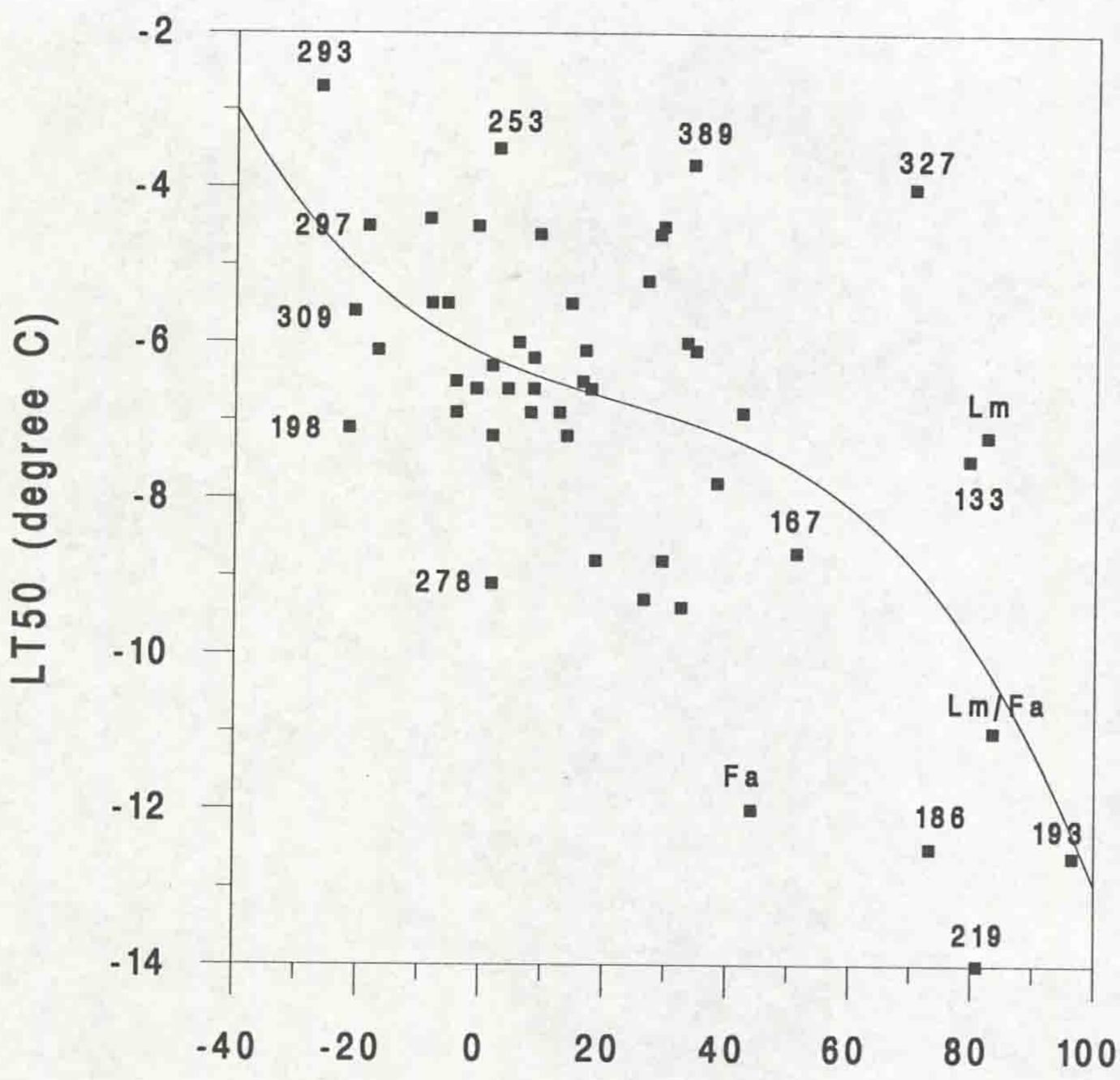


Figure 6.21. Relationship of tiller production index (TPI, %) in the field during the drought experiment (Cut 3) with freezing tolerance (LT_{50}) of ACD plants and the three control genotypes. Selected genotypes are labelled. Nos. (1-389) = ACD plants number, *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea* and *Lm/Fa* = Pentaploid hybrid.

The relationship between survival condition in the glasshouse drought experiment and freezing tolerance of ACD plants and the three control genotypes is illustrated in Figure 6.22 and 6.23. There was a weak but statistically significant negative correlation coefficient (r = -0.36, Appendix 6.9) between the survival score after 77 days drought and tolerance to freezing temperature.

The correlation coefficients between survival score after 44 days recovery following drought and freezing tolerance (r = -0.35, Appendix 6.9) were very similar with that seen at the end of drought.

There was a negative correlation between PGR under drought conditions in the field and cold tolerance (LT_{50}). This correlation was stronger during recovery following the drought period. Usually cold tolerant genotypes (e.g. Nos. 219, 193 and 186), with the exception of the fescue and pentaploid hybrid, had a higher yield under drought conditions than produced by cold sensitive genotypes (e.g. Nos. 293 and 253).

There was a weak but positive correlation between survival conditions under drought in the glasshouse and cold tolerance (LT_{50}). This correlation was weaker after recovery than at the end of drought.

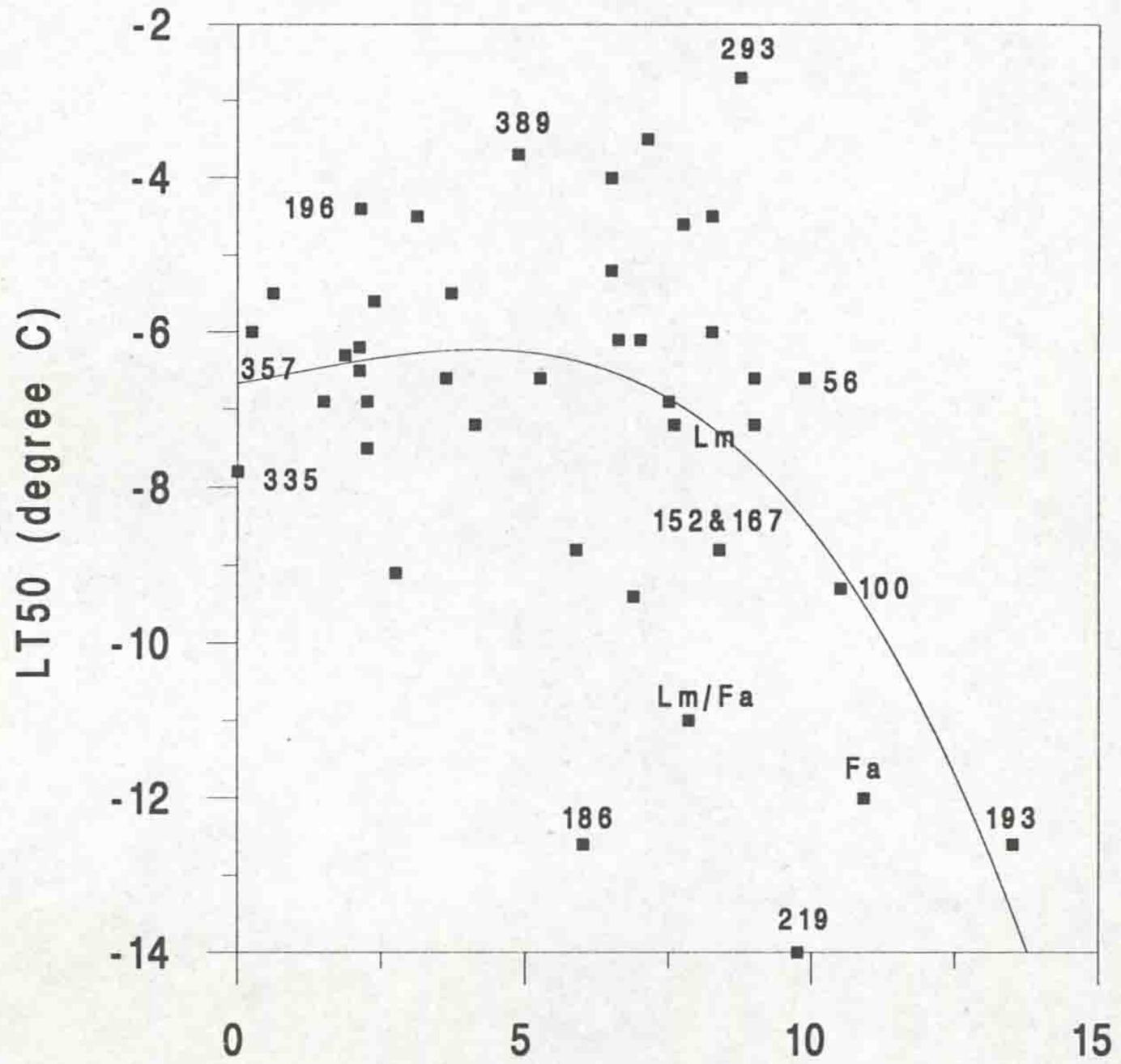


Figure 6.22. Relationship of survival score after 77 days drought in the glasshouse (scores 1-15) and freezing tolerance (LT_{50}) of ACD plants and the three control genotypes. Selected genotypes are labelled. Nos. (1-389) = ACD plants number, *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea* and *Lm/Fa* = Pentaploid hybrid.

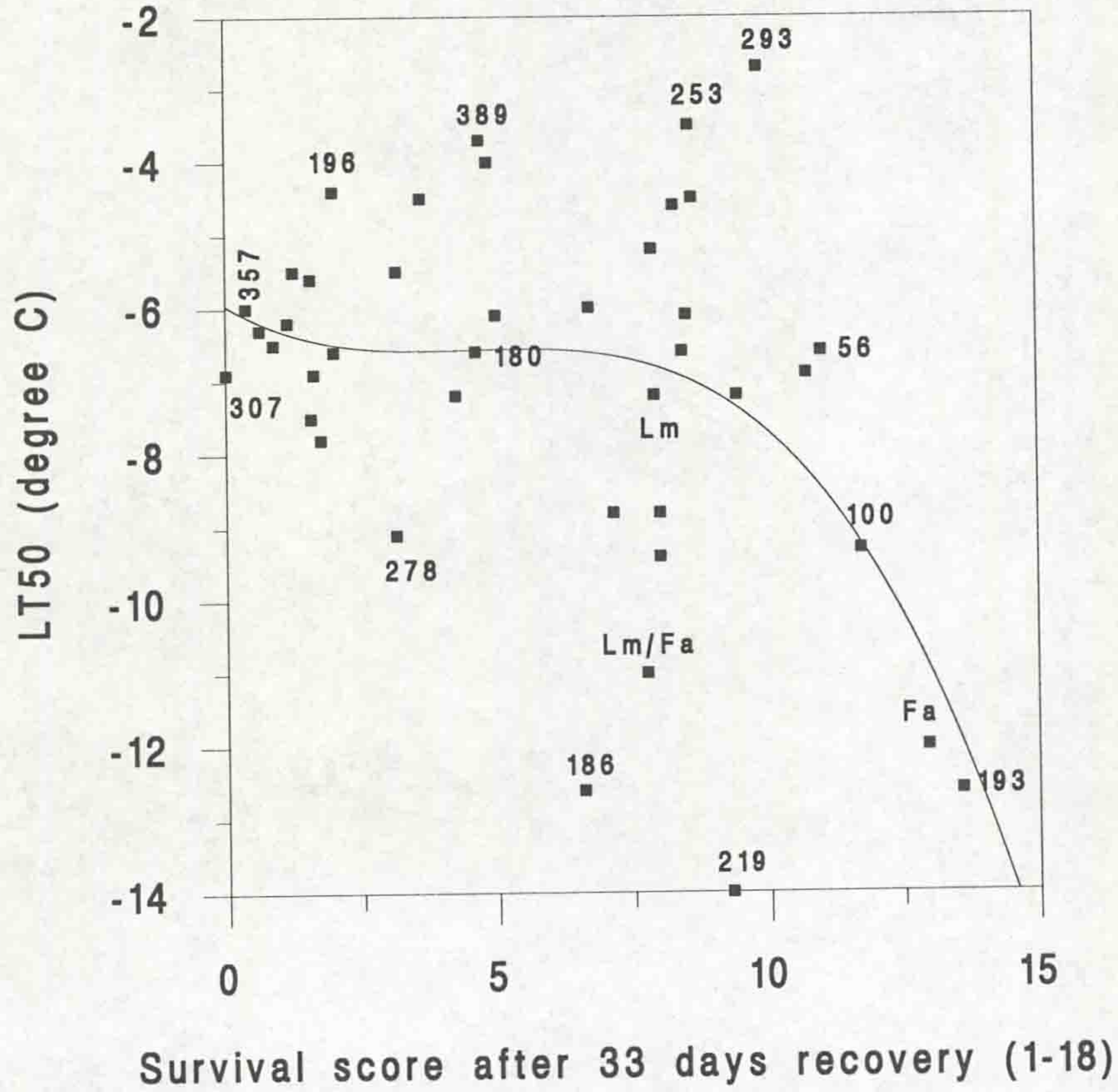


Figure 6.23. Relationship of survival score after 44 days recovery following drought in the glasshouse (scores 1-18) and freezing tolerance (LT_{50}) of ACD plants and the three control genotypes. Selected genotypes are labelled. Nos. (1-389) = ACD plants number, *Lm* = *L. multiflorum*, *Fa* = *F. arundinacea* and *Lm/Fa* = *Lm/Fa* pentaploid hybrid.

CHAPTER SEVEN

CHARACTERIZATION OF

PHYSIOLOGICAL AND MORPHOLOGICAL TRAITS

IN *L. MULTIFLORUM* × *F. ARUNDINACEA* PENTAPLOID

ANTHER CULTURE DERIVED PLANTS

7.1. Introduction

Selection to develop resistance to drought (and other environmental stress) in a breeding programme should be undertaken in the target environments within the region covered by the programme (Baker, 1989). The most reliable and productive forage grasses in a particular environment are often those adapted by natural selection or breeding to slightly harsher conditions (Henry Thomas, pers. comm.). Since drought resistance is affected by numerous factors (Taiz and Zeiger, 1991; Acevedo and Fereres, 1993; Richards, 1993 and Thomas, 1997), it may be necessary to use artificial environments. Such evaluation experiments under natural or modified environmental conditions (e.g. growth-chamber and glasshouse) have the advantage that they allow us to discriminate between different drought components, but have the disadvantage that they are expensive and take a long time. The number of plants that can be studied under those experiments is therefore limited.

The environmental stress resistance of plants may be determined or estimated by a wide range of physiological and morphological plant characters *in vitro* experiments and tests (Schulze and Hall, 1982; Saadalla, Shanahan and Quick, 1990; Premachandra *et al.*, 1991, Taiz and Zeiger, 1991; Monteith and Elston, 1993; Thomas, 1997). In view of the large number of ACD plants, I had to use techniques that were rapid and simple and permitted many samples to be evaluated.

7.2. Effects of desiccation on relative cell membrane damage (CMD) of *L. multiflorum* × *F. arundinacea* pentaploid anther culture derived plants

The ability of membranes to tolerate dehydration has been the subject of research for over 100 years (Levitt, 1980). When cells are severely dehydrated, turgor is lost and may or may not become negative. The protoplast shrinks, and the cell wall, if it is thin, may stay attached to the protoplast and collapse with it. If the

wall is thick the protoplast may become completely detached as it shrinks. The cell contents of damaged plasmalemma may leak out and the cell will die (Blum and Ebercon, 1981). The aim of this experiment was to identify the genetic diversity in tolerance of dehydration. The method used was to impose uniform desiccation stress on leaf tissue of as many genotypes as possible, and measure consequential membrane damage by quantifying the leakage of solutes from the tissues when incubated in de-ionized water.

7.2.1. Materials and methods

A total of 45 ACD plants plus two plants of *L. multiflorum*, two plants of *F. arundinacea* and one ramet of the original *L. multiflorum* × *F. arundinacea* pentaploid hybrid used in the field drought experiment, were incorporated in this test. All plants were trimmed, sprayed with pesticide and fungicide so as to produce healthy leaves then placed in a heated glasshouse to encourage a rapid growth rate. Leaf laminae were sampled on April 1995. The method of measuring the cell membrane damage is described in Chapter 2.

7.2.2. Results and discussion: cell membrane damage following desiccation

The target mean CMD was 50%, since this would give the best chance of distinguishing between genotypes. Actual mean CMD was 59% - encouragingly near the target value.

Analysis of variance of the effect of desiccation on cell membrane damage (%) is presented in Appendix 7.1. There was a highly significant ($P \leq 0.0001$) genotype effect on CMD - in other words, there were detectable and consistent differences between genotypes in tolerance to desiccation. Also, means differed on the two test days ($P \leq 0.0001$).

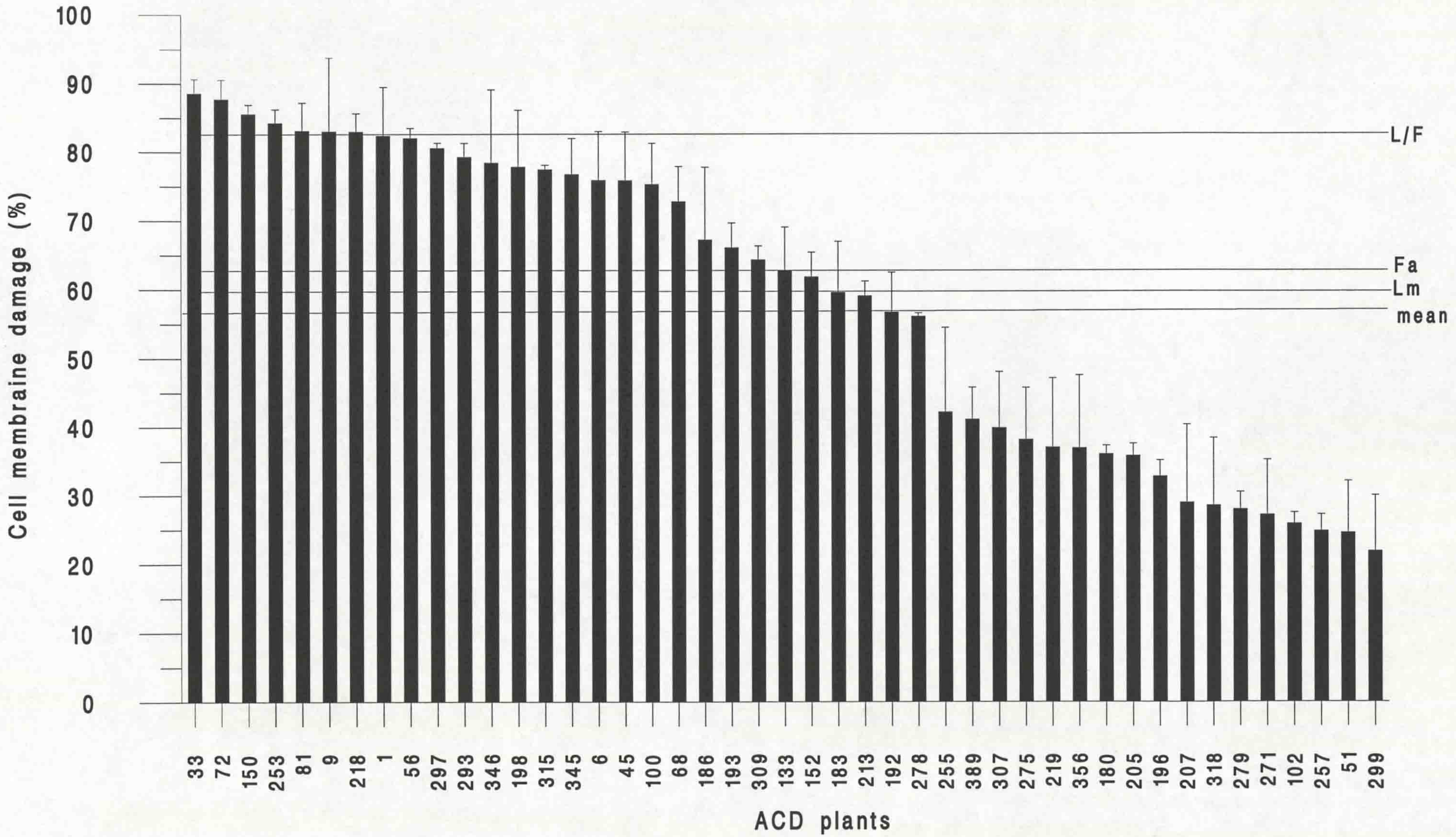
Inspection of the data on an individual day basis indicated that replicate observations were very similar. Nevertheless there was a significant genotype × day interaction indicating that genotypes performed differently on the two test days. It is difficult to explain the reasons for this in physiological terms and inspection of the treatment mean indicated that it was particularly associated with ACD plants 51, 102 and 133 which yielded low cell membrane damage on the second test day. This can only be put down to experimental error.

Figure 7.1 shows the large range in tolerance of cell membranes against the effect of desiccation, that is from 22% in the most tolerant plant (No. 299) to 88 % in the most sensitive plant (No. 33). CMD of 18 ACD plants exceeded the mean by 1 set, whilst 19 plants were less damaged. The cell membrane damage was similar (63%) in *L. multiflorum* (drought sensitive) and *F. arundinacea* (drought tolerant). This is in disagreement with Venkateswarlu and Ramesh (1993) who found that a drought tolerant groundnut (Kadiri-3) had less electrolyte leakage from leaf discs than a drought tolerant one (JL-24). The cell membrane of the *Lm/Fa* pentaploid hybrid (CMD = 82.8%) was less tolerant to desiccation than either *L. multiflorum* or *F. arundinacea* and there were only 4 ACD plants with slightly less membrane stability than the *Lm/Fa* pentaploid hybrid.

To explore the hypothesis that narrow leaves tend to be more xeromorphic, and therefore likely to be more tolerant to desiccation, the relationship between width and CMD was quantified. The mid-lamina width of irrigated ACD plants in the field averaged 6.76 mm and varied enormously, from 2.05 mm to 9.2 mm (see Chapter 4). There was, however no significant correlation between leaf width (mm) and cell membrane damage from desiccation ($r = -0.202$ with $P = 0.19$, Figure 7.2). There were plants with fine and narrow leaves (e.g. plants Nos. 1 and 56) and plants with broad leaves (e.g. Nos. 72 and 100) with equal damage after desiccation. Similarly in ACD plants with high CMD there were plants with wide leaves (e.g. plant Nos. 299 and 102) and plants with narrow leaves e.g. Nos. 271 and 257.

The relationship of percent cell membrane damage with transformed yield g per plant ($\log_{10}$ (x + 1)); and percent tiller production index under drought conditions under the polythene rain-out shelter is presented in Figure 7.3 to 7.6. The cell membrane of leaves of drought tolerant plants (e.g. No. 193 and 219 and *Lm/Fa* pentaploid hybrid) and also drought sensitive plants (e.g. plants Nos. 196, 213, 309, 346 and 297) all showed different reaction to desiccation. In addition, ACD plants with the same cell membrane damage (e.g. plants Nos. 196, 205, 180, 356 and 219 or plants Nos. 309, 183, 152, 133, 193 and *F. arundinacea*) had different degrees of drought tolerance following 87 days drought (Figure 7.3). Similar results were obtained between the percent cell membrane damage with the yield at the end of the drought; and after recovery following drought (Figures 7.4 and 7.5). Furthermore the actual data from the field drought treatment (Appendix 7.2) showed a correlation between the plant growth rate (PGR, mgd^{-1}) and tiller production capacity (%) at the field with cell membrane damage (%). No correlation was found between PGR and cell membrane damage; and neither was there a correlation between them, when the plants with zero yield were ignored (Appendix 7.2). As Figure 7.6 shows, among ACD plants with cell membranes sensitive to desiccation, there were plants with either very high tiller production index (e.g. 193, 133 and 186), moderate tiller production index (e.g. Nos. 33, 72, 1 and 9), and even negative tiller production index (e.g. Nos. 293, 198 and 309). Among ACD plants with membranes tolerant to desiccation, there were plants with high tiller production index (e.g. No. 219), moderate tiller production index (e.g. Nos. 299, 257, 318, 205, 289 and 255), and tiller production index about zero (e.g. Nos. 275, 279 and 196). The correlation coefficients between these two characters was r = -0.076 and P = 0.607 (Appendix 7.2).

Figure 7.1. The effect of desiccation on cell membrane damage of ACD plants. Each point is mean of 4 samples. Nos. (1-389) = ACD plants, Lm = *L. multiflorum*, Fa = *F. arundinacea* and Lm/Fa = *L. multiflorum* × *F. arundinacea*. Error bars are one standard error of the mean.


Cell membraine damage (%)
100
90
80
70
60
50
40
30
20
10
0
L/F
Fa
Lm
mean
33
72
150
253
81
9
218
1
56
297
293
346
198
315
345
6
45
100
68
186
193
309
133
152
183
213
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51
299
ACD plants

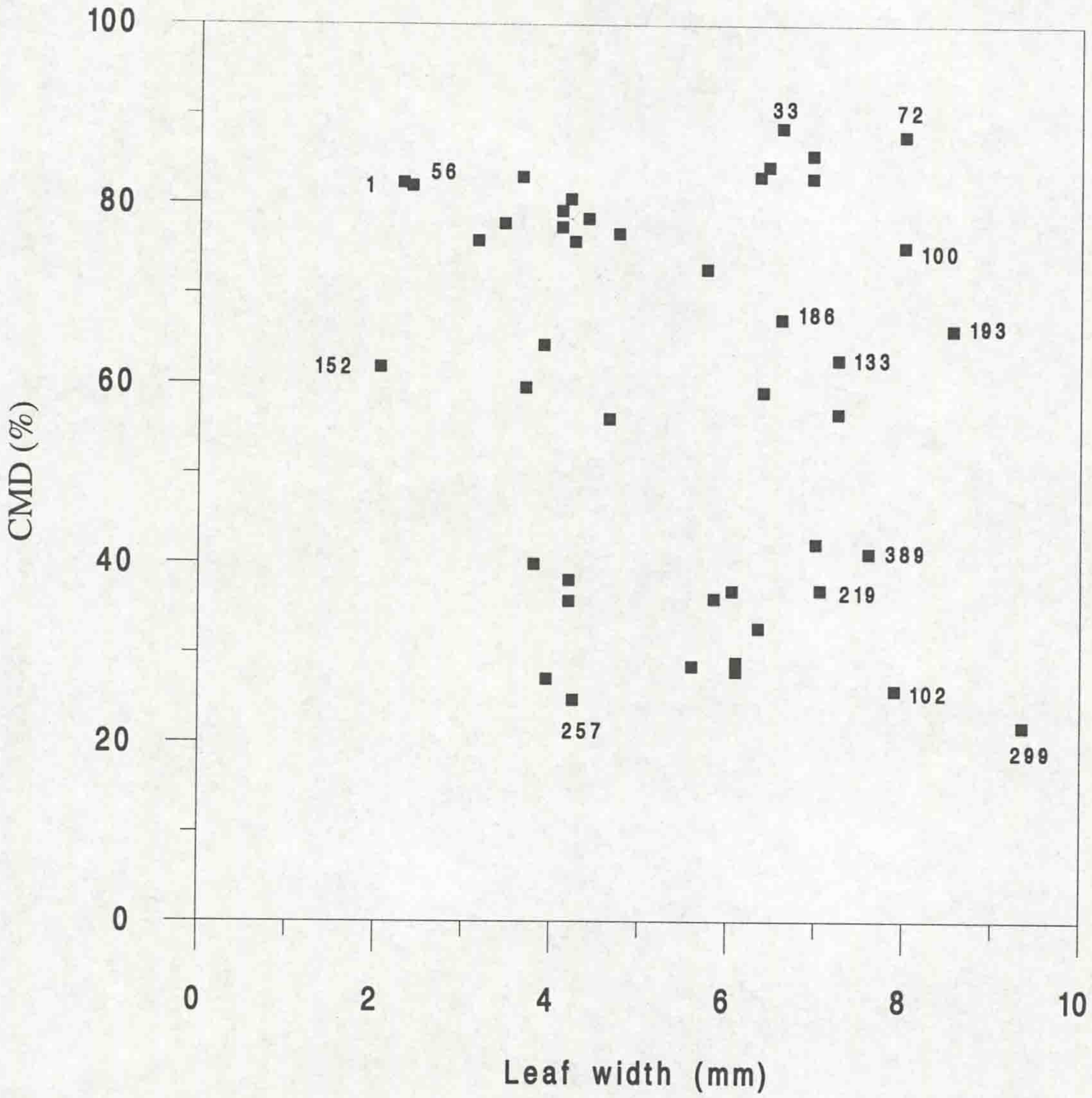


Figure 7.2. Relationship of leaf width (mm) in the field with the cell membrane damage (%). Selected plants are labelled. CMD = cell membrane damage, Nos. (1-389) = ACD plants, Lm = *L. multiflorum*, Fa = *F. arundinacea* and Lm/Fa = *L. multiflorum* × *F. arundinacea*.

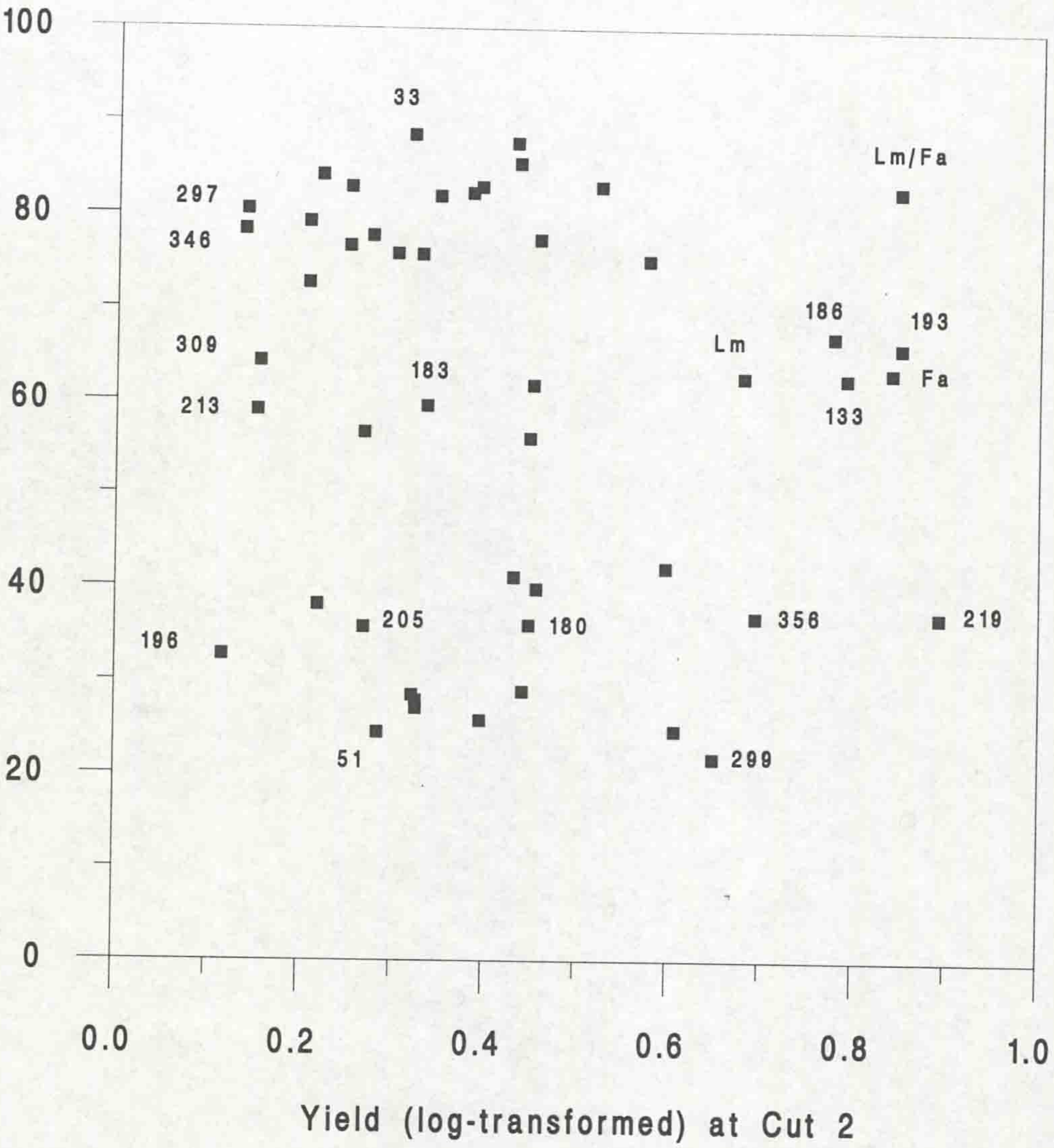


Figure 7.3. Relationship of log-transformed yield ($\log_{10}$ (x + 1)) of Cut 2 (= yield of 87 days during drought in the field) with the cell membrane damage (%). Selected genotypes are labelled. CMD = cell membrane damage, Nos. (1-389) = ACD plants, Lm = *L. multiflorum*, Fa = *F. arundinacea* and Lm/Fa = *L. multiflorum* × *F. arundinacea*.

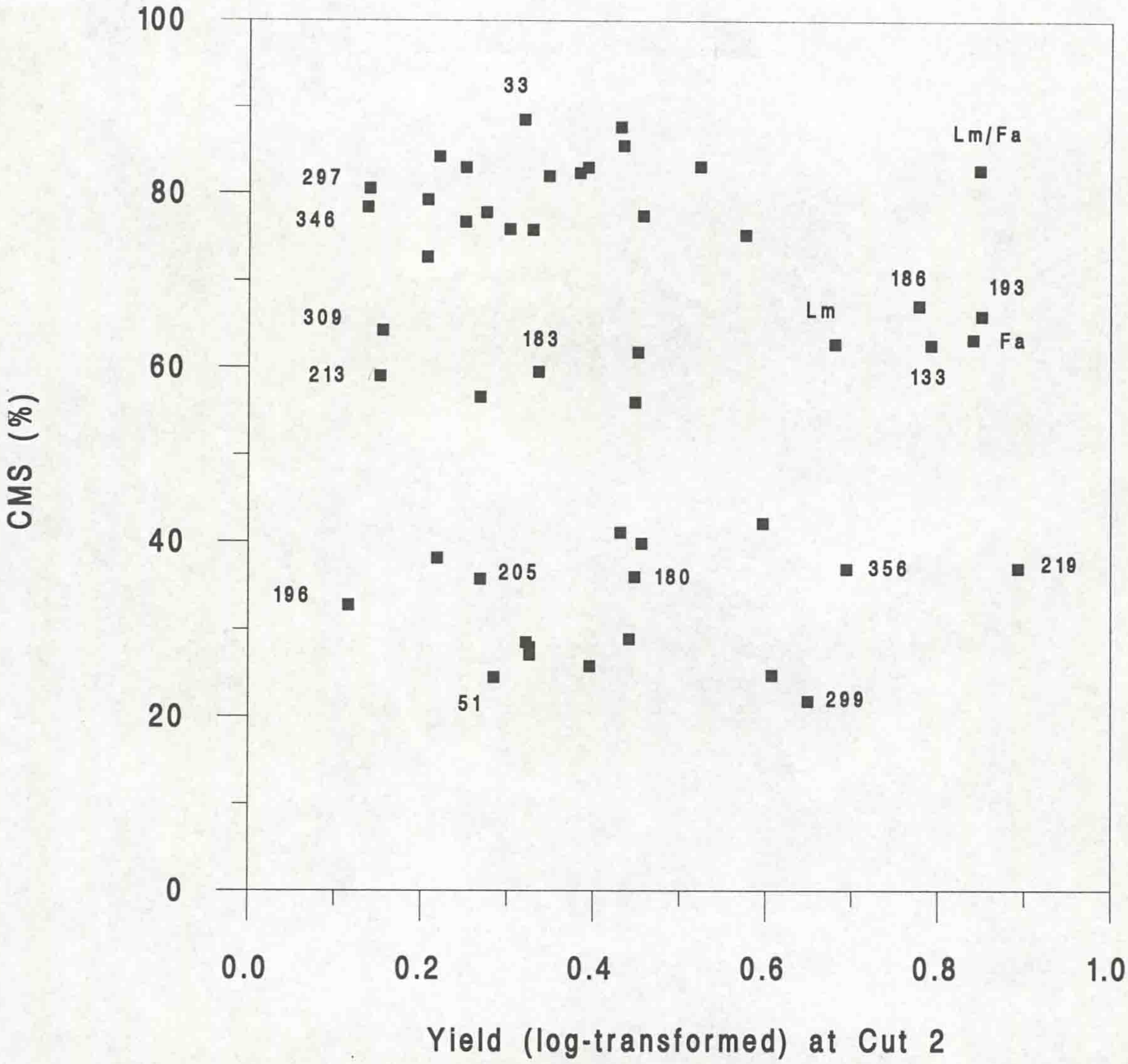


Figure 7.4. Relationship of log-transformed yield ($\log_{10}$ (x + 1)) of Cut 3 (= yield of 18 days at the end of drought in the field) with the cell membrane damage (%). Selected genotypes are labelled. CMD = cell membrane damage, Nos. (1-389) = ACD plants, Lm = *L. multiflorum*, Fa = *F. arundinacea* and Lm/Fa = *L. multiflorum* × *F. arundinacea*.

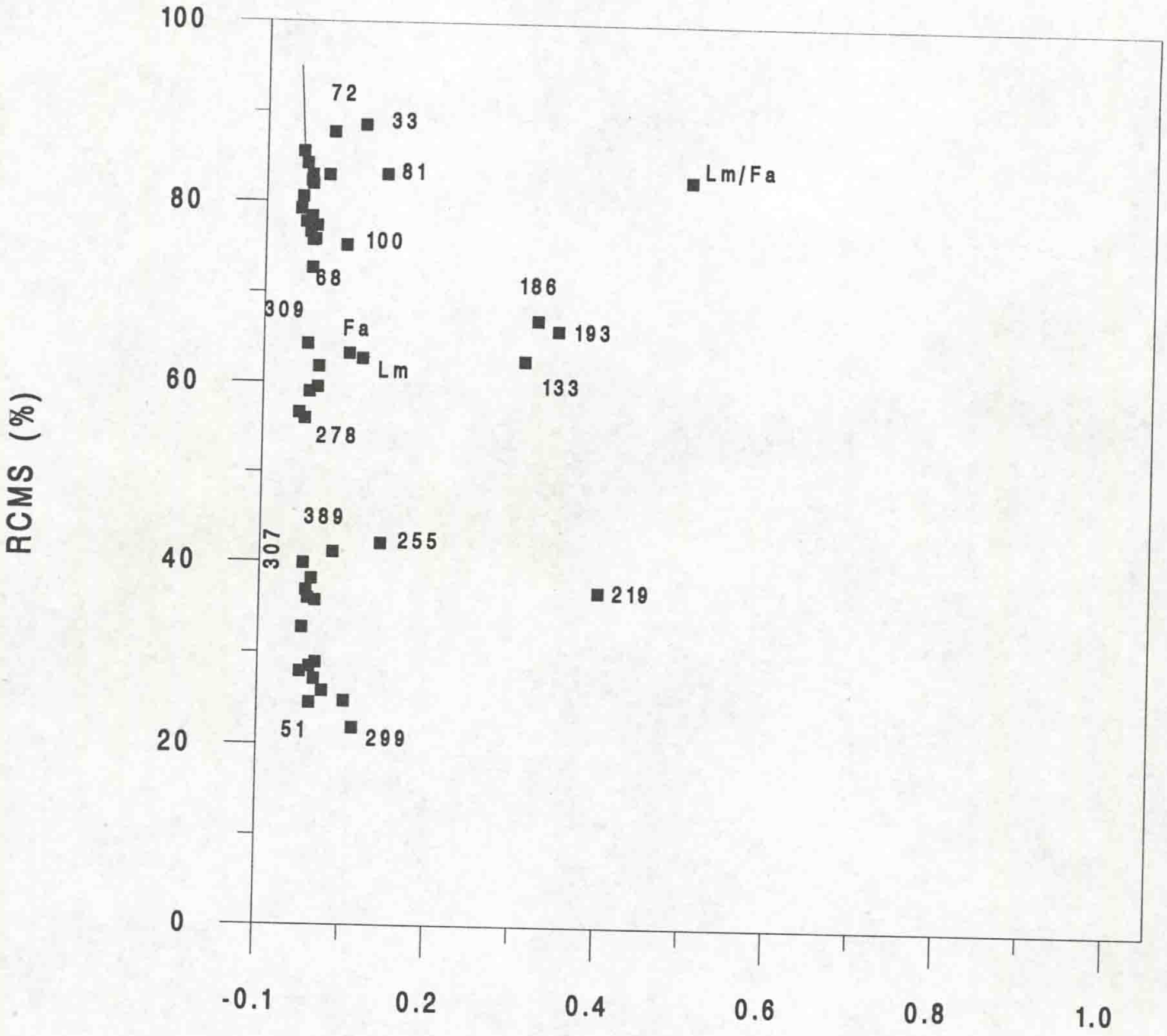


Figure 7.5. Relationship of log-transformed yield ($\log_{10}$ (x + 1)) of Cut 4 (= yield of 44 day recovery following drought in the field) with the cell membrane damage (%). Selected genotypes are labelled. CMD = cell membrane damage, Nos. (1-389) = ACD plants, Lm = *L. multiflorum*, Fa = *F. arundinacea* and Lm/Fa = *L. multiflorum* × *F. arundinacea*.

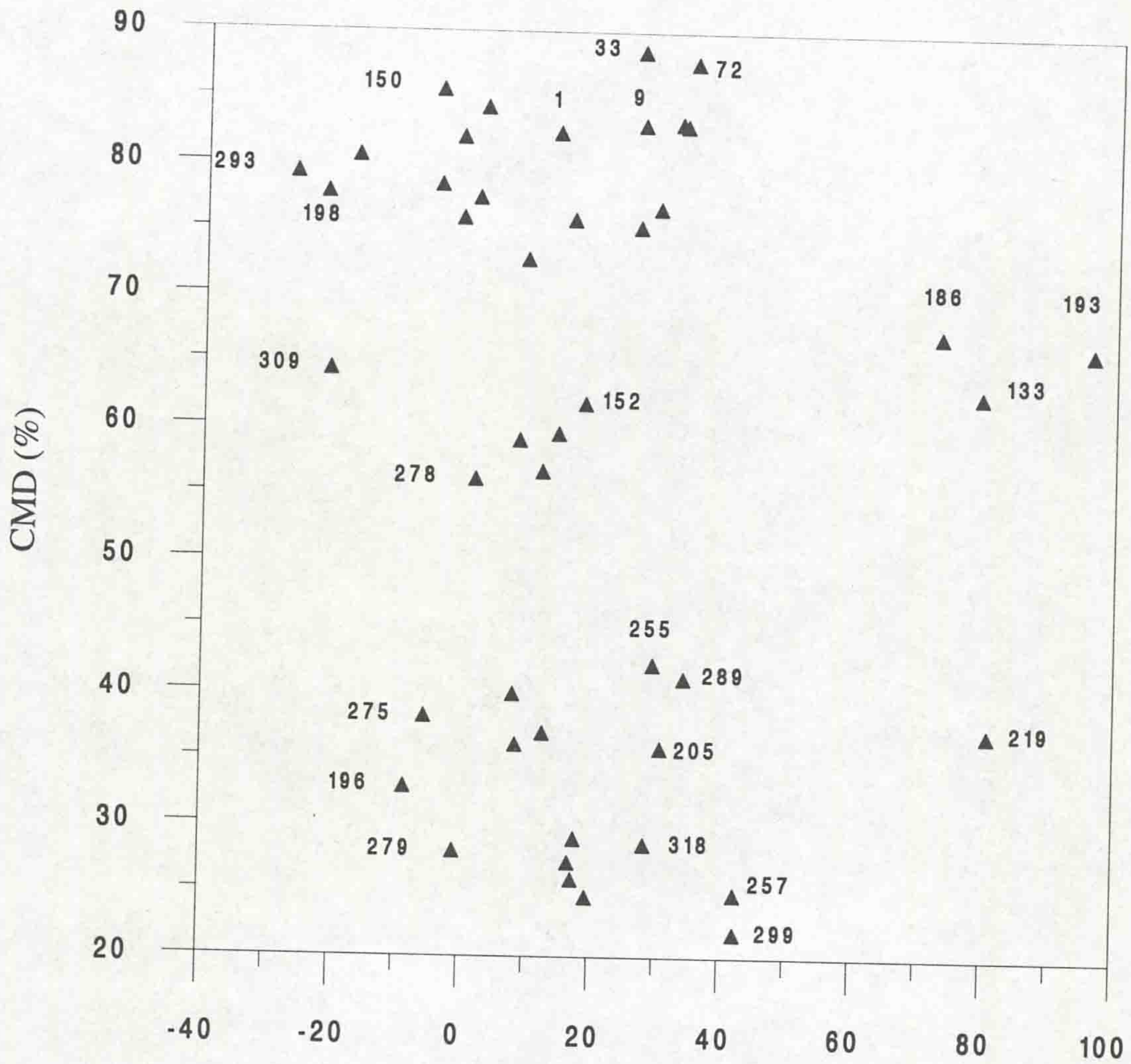


Figure 7.6. Relationship of tiller production capacity index (TPC) (%) in the field with the cell membrane damage (%). Selected genotypes are labelled. CMD = cell membrane damage, Nos. (1-389) = ACD plants, Lm = *L. multiflorum*, Fa = *F. arundinacea* and Lm/Fa = *L. multiflorum* × *F. arundinacea*.

The practical aim of determining genetic differences in CMD is that they can be used as a basis for evaluating and selecting drought-resistant germplasm. To test this proposal, correlations were sought between CMD and performance of spaced plants under drought in the glasshouse.

The relationship between survival score of droughted ACD plants in a glasshouse at the end of the drought period and after recovery following the drought, with the percentage of cell membrane damage is shown in Figures 7.7 and 7.8. Among plants with cell membranes sensitive to desiccation, there were plants which died (e.g. No. 33), showed only poor survival (e.g. No. 150), good survival score (e.g. Nos. 72, 81, 253, 218 and *Lm/Fa* pentaploid hybrid) and very good survival score (e.g. Nos. 193, *F. arundinacea*, 100 and 56). Also among the ACD plants with membranes tolerant to cell desiccation, there were plants (e.g. Nos. 219, 279 and 257) with good survival scores, moderate survival scores (e.g. Nos. 389, 255 and 180) and plants with only a trace of survival (e.g. Nos. 299 and 275) at the end of drought period (Figure 7.7). No relationship was found between high or low CMD scores and regrowth in recovery following the drought (Figure 7.8). There was no correlation between survival score and cell membrane damage either at the end of drought ($r = 0.213$ with $P = 0.194$) or after recovery following drought ($r = 0.238$ with $P = 0.145$) .

The cell membrane stability test has been widely used in barley (Bandurska and Gniazdowska-Skoczek, 1995), wheat (Blum and Ebercon, 1981; Premachandra and Shimada, 1988, Saadalla, Quick and Shsnshsn, 1990 and Ashraf, Khan and Azmi, 1992), maize (Saadalla, Saneoka and Quick 1990 and Saadalla, Quick and Shsnshsn 1990) sorghum (Premachandra, Saneoka Fujita and Ogata, 1992) and many other crops. Cell membrane damage in all the cases was measured at different drought severity, usually established by incubating in polyethylene glycol (PEG) solution, rather than by exposure to dry air as used here. In some cases higher membrane stability (less cell membrane damage) could be correlated with better field performance. For example Premachndra *et al.* (1991) discriminated drought

tolerance of eight maize cultivars by percentage injury caused under increasing water deficits in the PEG test. Ashraf, Khan and Azmi (1992) unlike earlier researchers (Blum and Ebercon, 1981) considered that measurement of cell membrane stability by the PEG test is a reliable method for measuring drought tolerance in wheat. They found correlation between cell membrane stability and leaf water potential, osmotic potential, water loss from excised leaf tissue, and dry weight of plant. In barley the injury to cell membranes was markedly influenced by leaf age, leaf position on the stem, and the degree of drought stress (Bandurska and Gniazdowska-Skoczek, 1995). They also found differences in percentage of injury to cell membrane between the barley genotypes.

Acclimation to drought can confer cold hardiness on grasses (Thomas and James, 1993). The hypothesis was raised that genotypes having tolerance to desiccation might also be tolerant to cold. The relationship between freezing tolerance test of the tillers (LT_{50}) and cell membrane damage is shown in Figure 7.9. There was no clear relationship between these characters. The cell membranes from leaf tissue with the highest (Genotype 219), and lowest (e.g. Nos. 196 and 389) tolerance to freezing temperatures, both displayed good tolerance to desiccation. Among the genotypes which were cell membrane sensitive to desiccation, were the cold tolerant *Lm/Fa* pentaploid hybrid (LT_{50} = - 11°C), and cold sensitive plants (e.g. Nos. 293 and 253) with an LT_{50} around -3°C. The freezing tolerance of the most sensitive plant (No. 72) and the most tolerant plant (No. 257) to desiccation of the cell membrane were similar (LT_{50} between -6 to -7°C). Among the genotypes tolerant to freezing temperature, there was plant 219 (with CMD = 37.0%), plants Nos. 193 and 186 and *F. arundinacea* (CMD between 63 and 67%) and *Lm/Fa* pentaploid hybrid with CMD = 82.8%.

It should be emphasised that desiccation tolerance is only one means whereby plants are drought resistant and in apical and meristematic tissues there may be survival mechanisms to prevent catastrophe (Turner,1979).

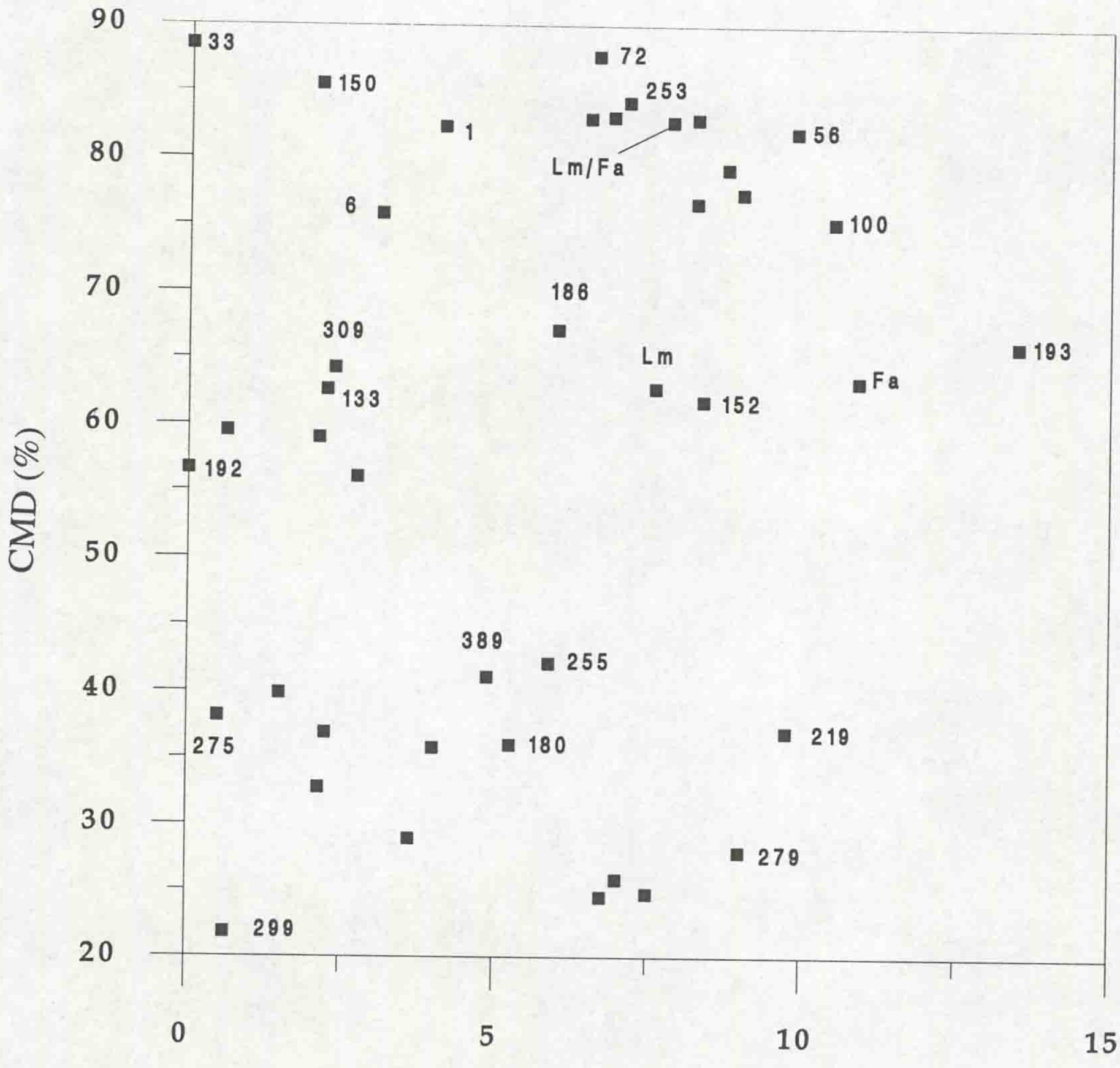


Figure 7.7. Relationship of survival score at the end of drought (77 days) (0-15, see Table 6.8) in a glass house with the cell membrane damage (%). Selected genotypes are labelled. CMD = cell membrane damage, Nos. (1-389) = ACD plants, Lm = *L. multiflorum*, Fa = *F. arundinacea* and Lm/Fa = *L. multiflorum* × *F. arundinacea*.

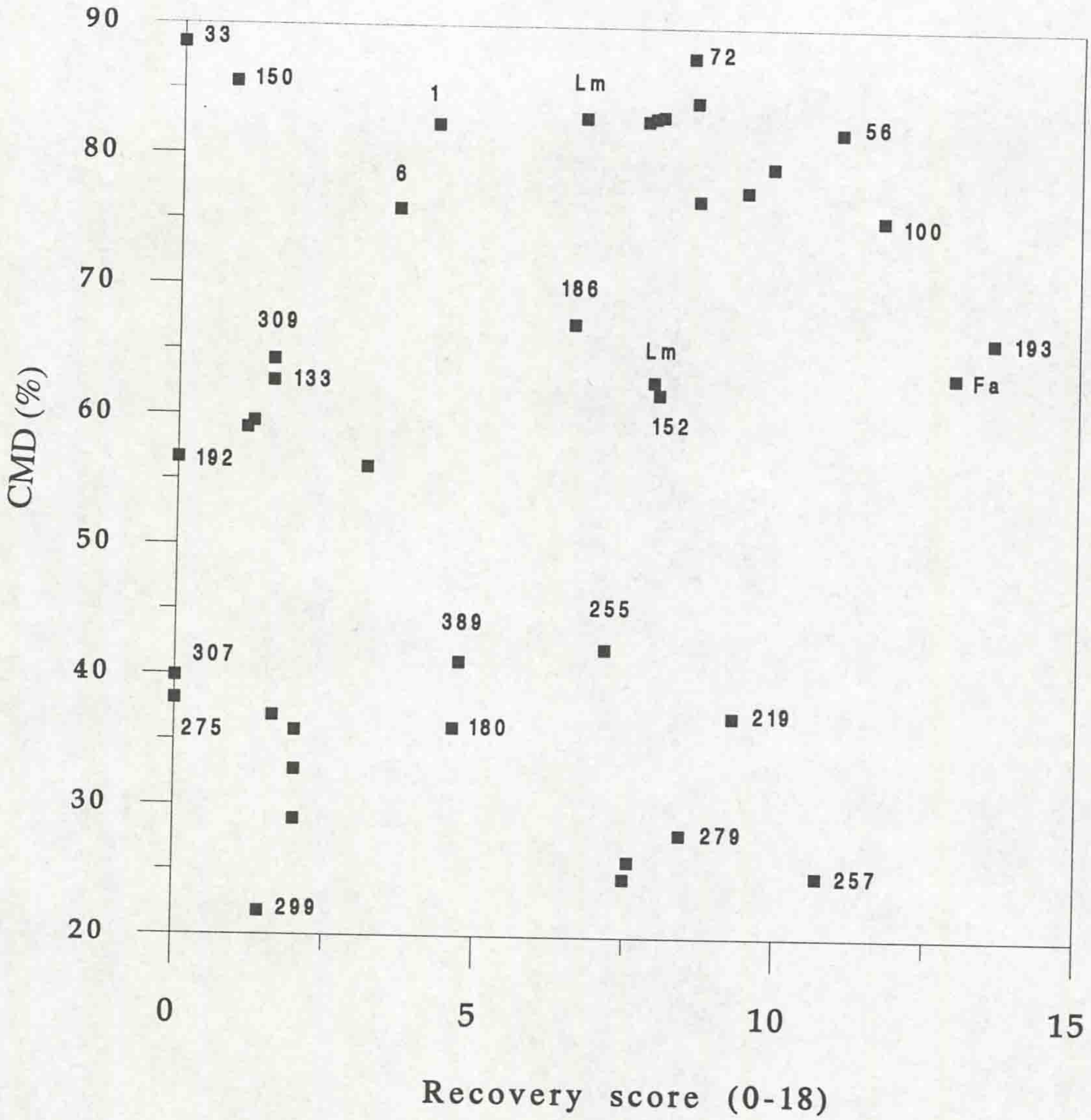


Figure 7.8. Relationship of survival score after 33 days recovery following drought (0-18, see Table 6.8) in a glass house with the cell membrane damage (%). Selected genotypes are labelled. CMD = cell membrane damage, Nos. (1-389) = ACD plants, Lm = *L. multiflorum*, Fa = *F. arundinacea* and Lm/Fa = *L. multiflorum* × *F. arundinacea*.

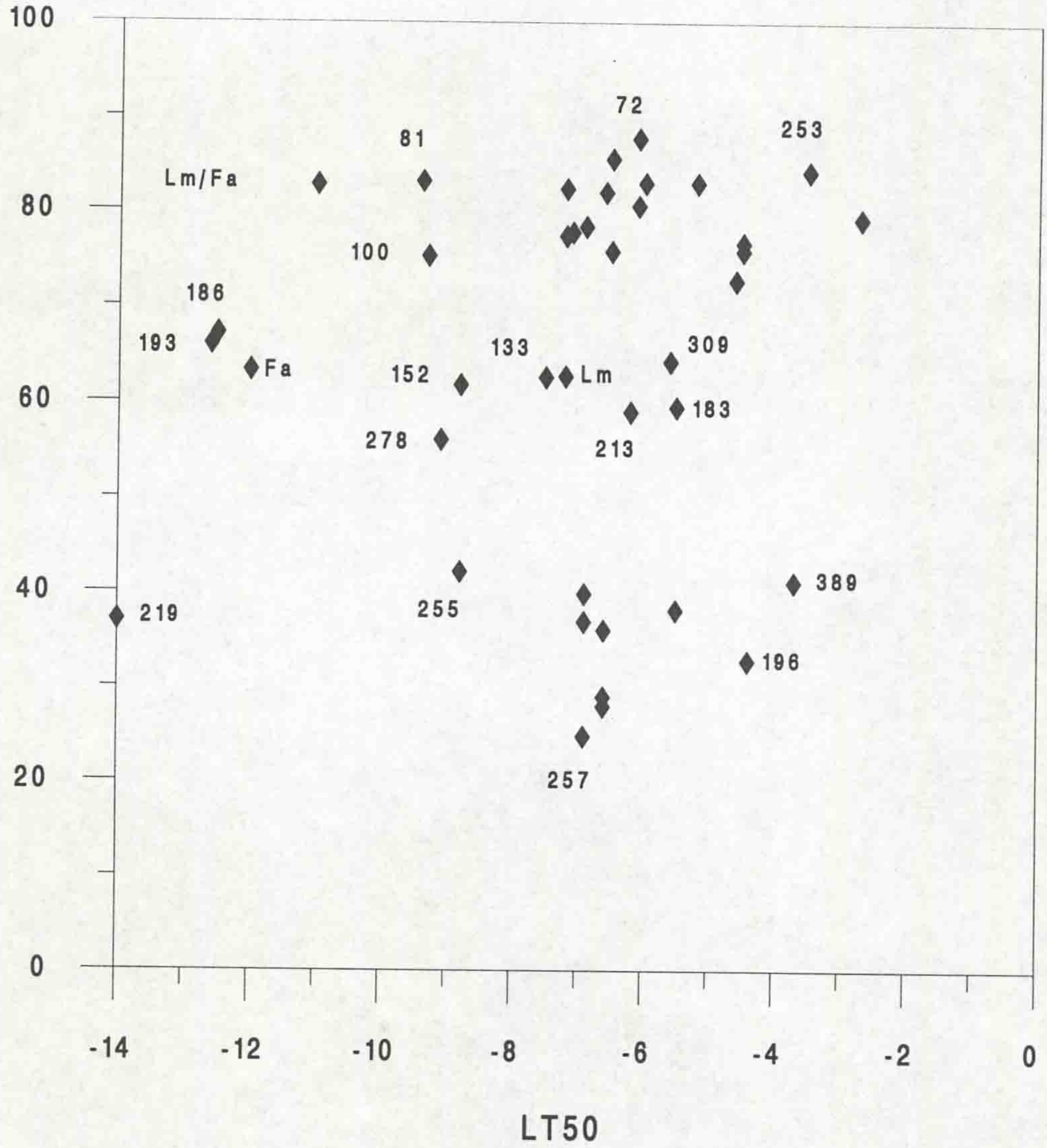


Figure 7.9. Relationship between freezing tolerance and the cell membrane damage (%). Selected plants are labelled. CMD = cell membrane damage, LT_{50} = the lethal temperature of 50% kill values, Nos. (1-389) = ACD plants, Lm = *L. multiflorum*, Fa = *F. arundinacea* and Lm/Fa = *L. multiflorum* × *F. arundinacea*

7.3. Diversity of stomatal density on adaxial and abaxial leaf surfaces of *L. multiflorum* × *F. arundinacea* pentaploid anther culture derived plants

There are three important variables in the movement of water from the internal leaf tissues to the atmosphere. The resistance associated with diffusion through the stomatal pores is the most important followed by the resistance of the leaf cuticle to water loss. Finally, there is the resistance due to the layer of unstirred air next to the air surface through which water vapour must diffuse to reach the turbulent air of the atmosphere (Taiz and Zeiger, 1991). In this work only stomatal resistance was considered.

Yamashita *et al.* (1995) indicated that two genes controlled stomatal frequency in the F_1 and F_2 rice generations. However environmental and nutrition factors e.g. CO_2 concentration and irrigation in groundnut (Clifford *et al.*, 1995), CO_2 concentration in *Egyptian Olea europeea* (Beerling and Chaloner, 1993), Fe in wheat (Sharma, *et al.*, 1994), temperature in cotton (Lu and Zeiger, 1994) and Gamma-ray in grapevine (Lima-da-Silva and Doazan, 1995) influenced stomata frequency.

The stomata that occur in leaves, and in the stem of some species of plants, are the major pathway for gaseous exchange between plant and atmosphere. Carbon dioxide is taken in through stomata for use in photosynthesis, while water vapour passes out into the atmosphere. Stomata have dual function: open stomata permit photosynthesis by exchange O_2 and CO_2 and evaporation cools the leaf; closed stomata conserve water under drought (Clarke and Durley, 1981). As drought develops photosynthesis declines (Jones, Leafe and Styles, 1980 and McCree, 1986) but in terms of adaptation it is clear that plants can conserve water by partly closing stomata (Monteith and Elston, 1993). The main limitation of photosynthesis during the early stages of drought is due to stomatal closure (Chaves, 1991).

The likely effects of increasing dehydration are (Kaiser, 1987):

- RWC (relative water content) 100 - 70%: Reduction in photosynthesis due to stomatal closure, which is rapidly reversible.

- RWC 70 - 35%: In bright light, photosynthetic capacity is reduced, and recovers only slowly on rehydration.

- RWC < 30%: Irreversible decrease in photosynthetic capacity, leading to death, due to membrane damage in the chloroplast.

The rate of gas exchange (O_2, CO_2 and water vapour) between air and leaf depends on the size and distribution of stomata (Clarke and Durley, 1981). The aim of this piece of work was firstly to determine the density and frequency of stomata on the ad- and abaxial of leaf surfaces. The second aim was to find out possible relationship of stomatal distribution with environmental stresses, i.e. drought resistance and possibly cold tolerance.

7.3.1. Materials and methods

Stomatal frequency and distribution on the upper (adaxial) and lower (abaxial) leaf surfaces were determined for a total of 17 ACD plants and the three relevant control genotypes *L. multiflorum*, *F. arundinacea* and *L. multiflorum* × *F. arundinacea* pentaploid hybrid as explained in Chapter 2.

7.3.2. Results and discussions: Stomatal frequencies and distribution

Stomata were found on both leaves surfaces of all ACD plants investigated. Distribution of stomata on the adaxial leaf surface was different to those on the abaxial leaf surface (Figure 7.10 and 7.11). In general stomata on abaxial leaf surface were more easily discerned than on the adaxial surface. Stomata usually were seen two rows in each group on the abaxial surface, while there were more rows (3-4) mainly at the edges of leaves on the adaxial leaf surface. The rows of stomata in the ACD plants were closer on the adaxial than on the abaxial. The

frequency of stomata in the two parallel rows within one group of stomata sometimes were not equal, e.g. on the abaxial leaf surface on plant No. 177 (Figure 7.11, bottom).

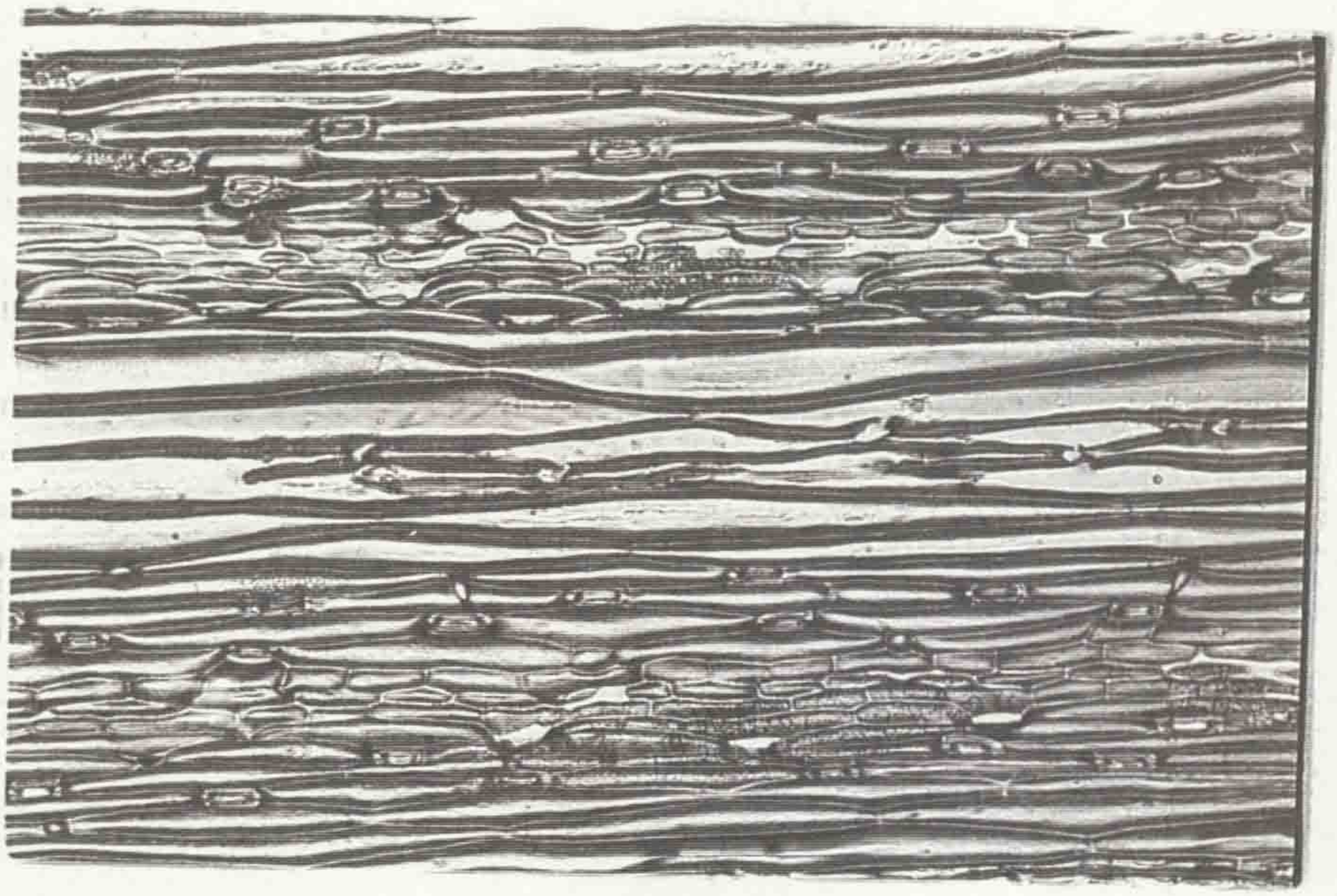
The two parent species had almost identical stomatal frequency on the adaxial leaf surfaces, but *F. arundinacea* had higher stomatal frequency on the abaxial surface than did *L. multiflorum*. *L. multiflorum* was almost amphistomatous, with approximately equal numbers of stomata on the ab- and adaxial leaf surfaces. *F. arundinacea* was hypostomatous, having more stomata on the abaxial leaf surface. By contrast, the *Lm/Fa* pentaploid hybrid was strongly hyperstomatous, having three times as many stomata on the adaxial leaf surface as on the abaxial (Table 7.1).

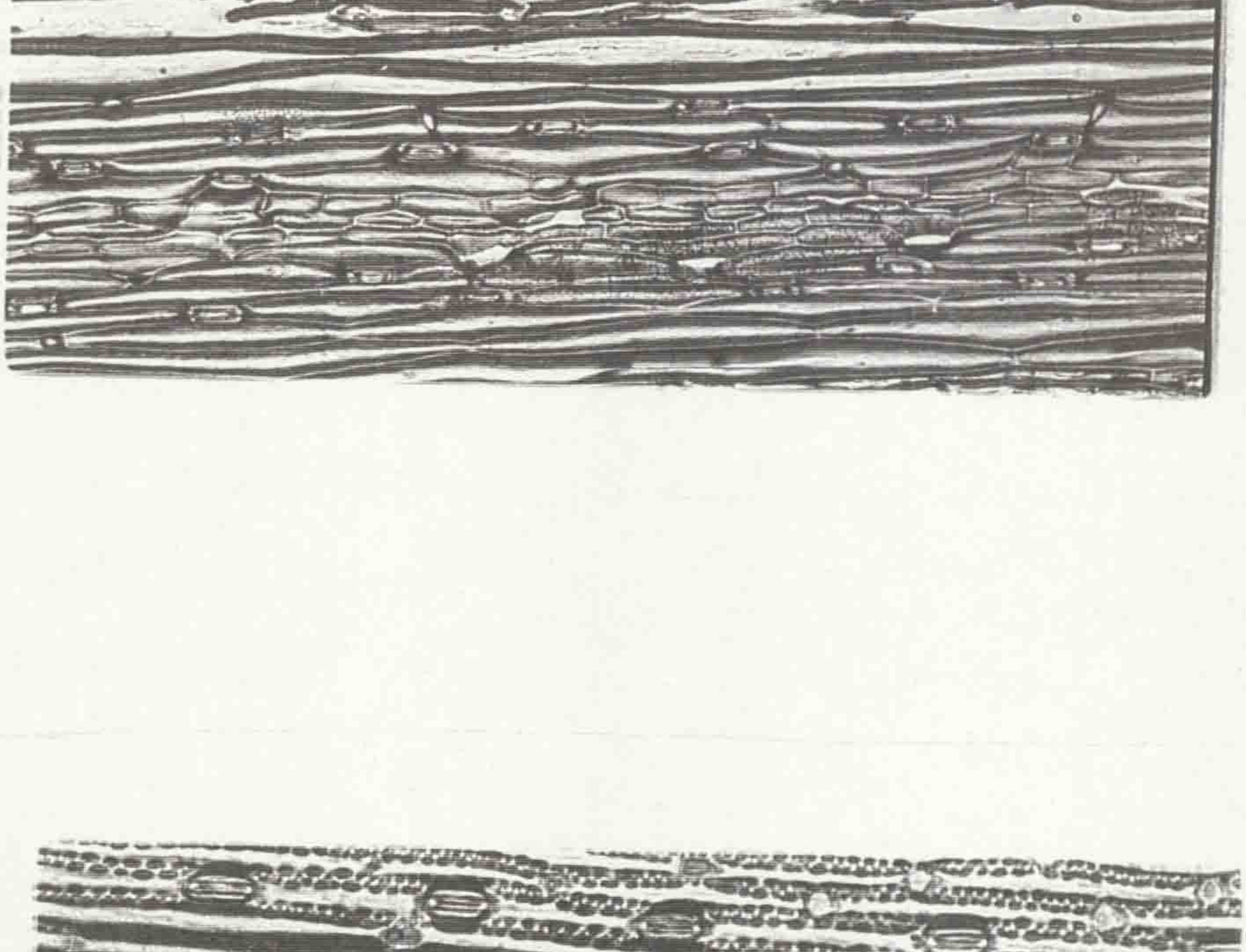
The ACD plants exhibited even greater diversity in stomatal frequency and distribution (Table 7.1). Adaxial stomatal frequency ranged from 69 to 192 per mm^2, or 53% to 146% of that in the *Lm/Fa* pentaploid hybrid from which they were derived. Abaxial stomatal frequency ranged from 19 to 111 per mm^2, or 49% to 282% of *Lm/Fa* pentaploid hybrid values. Stomatal distributions varied from amphistomatous (in ACD plant No. 74) to strongly hyperstomatous (e.g. ACD No. 177, Figure 7.11), and consequently there was no correlation between stomatal frequencies on adaxial and abaxial surfaces.

Table 7.1. Stomata frequency on adaxial (upper) and abaxial (lower) leaf surfaces and the adaxial : abaxial ratio of ACD plants and their control genotypes. (Stomata/mm^2, O = Observation, Es= Estimation, and χ^2 ($\alpha = 0.01$, df = 19) = 36.19)

	Observation			
Genotypes	Adaxial	Abaxial	Ad. + Ab.	Ad./Ab.
ACD 2'	56.2	34.6	90.8	1.62
ACD 6	104.4	20.4	124.8	5.12
ACD 32	60.7	40.6	101.3	1.50
ACD 38	62.4	14.3	76.7	4.36
ACD 51	87.1	41.8	128.9	2.08
ACD 54	38.3	32.9	71.2	1.16
ACD 68	106.5	54.8	161.3	1.94
ACD 74	57.8	61.5	119.3	0.94
ACD 82	72.4	32.8	105.2	2.21
ACD 84	39.3	25.0	64.3	1.57
ACD 133	85.2	44.3	129.5	1.92
ACD 177	91.3	10.7	102.0	8.53
ACD 186	78.3	42.2	120.5	1.86
ACD 193	60.9	37.4	98.3	1.63
ACD 194	49.1	38.3	87.4	1.28
ACD 219	59.8	31.1	90.9	1.92
ACD 271	45.7	43.2	88.9	1.06
L. multiflorum	33.5	36.1	69.6	0.93
F. arundinacea	33.3	45.8	79.1	0.73
Lm/Fa (5x) hybrid	72.8	21.8	94.6	3.34
Average	64.75	35.48	100.23	2.29
$\chi^2 = \Sigma(O-E)^2/E$	144.40	94.29	114.72	30.06

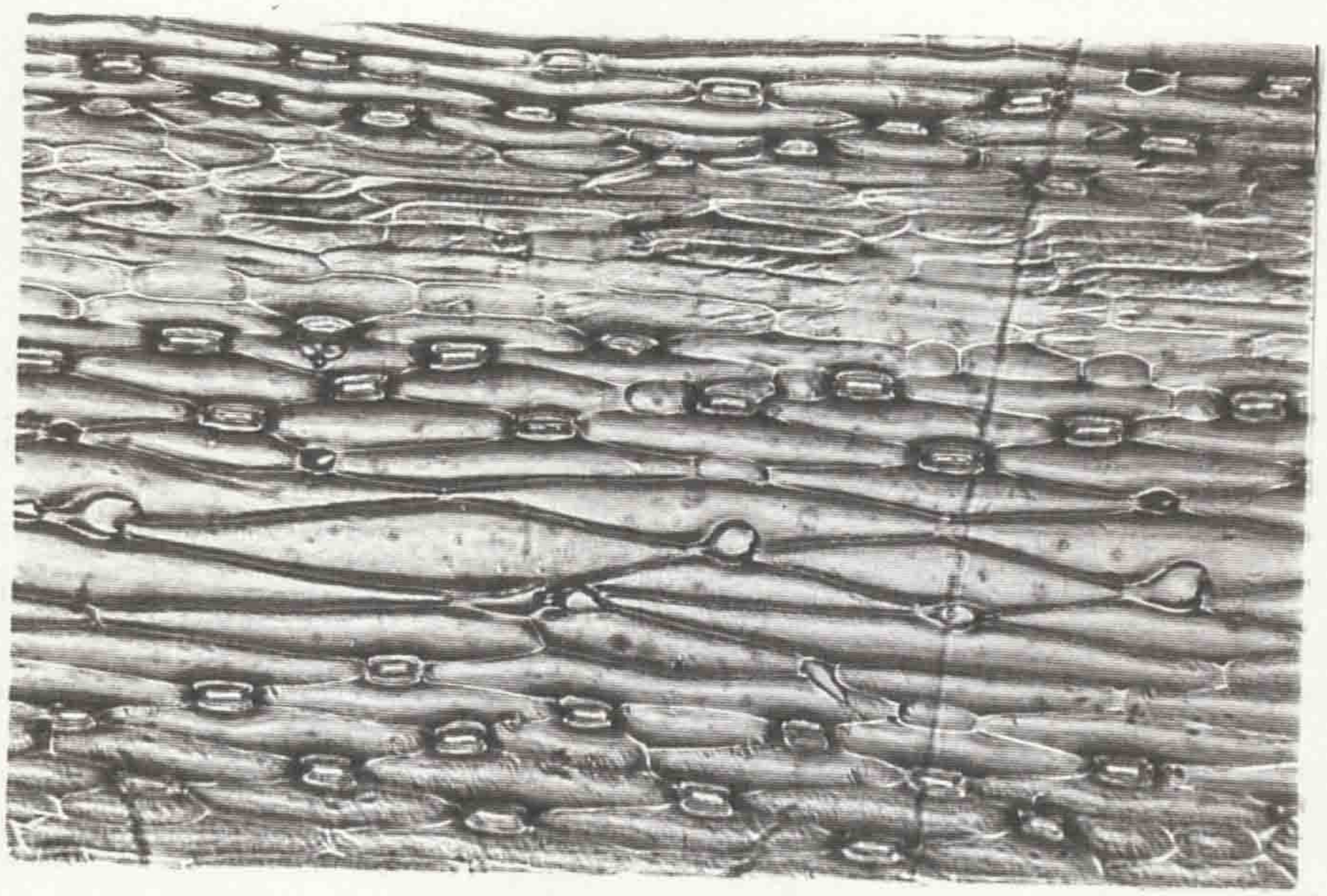
Figure 7.10. Stomatal frequency and distribution on newly fully expanded laminae of ACD plant 219. ACD plant 219 had the highest yield under drought condition in the field experiment and was the most cold tolerant plant in the freezing test. The adaxial surface (upper Figure) had more stomata than the abaxial (adaxial : abaxial = 1.92) but they were smaller and relatively irregular. The abaxial surface (bottom Figure) had less stomata but these were larger, and relatively regular (magnification was 10 × 10X)

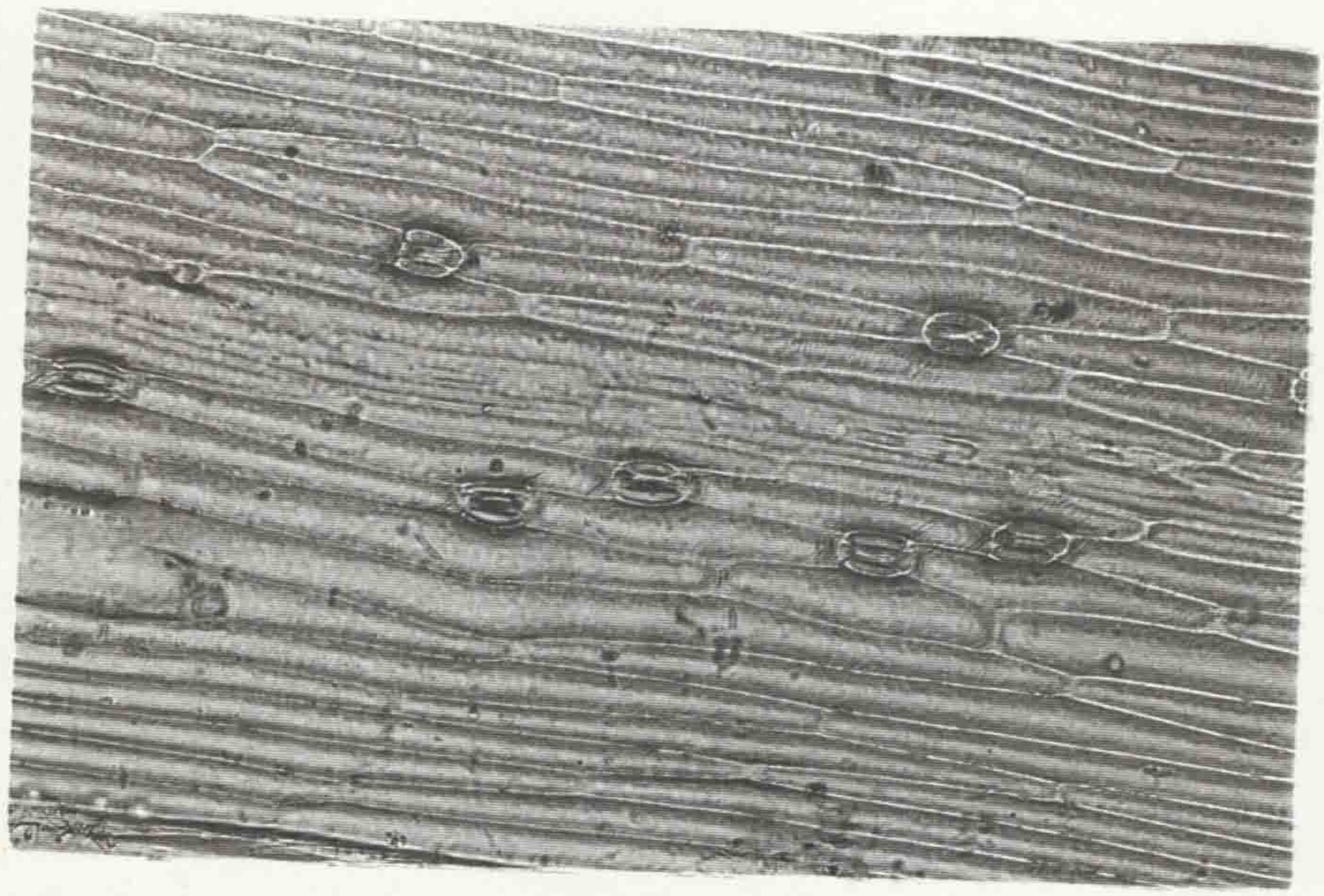




200a.

Figure 7.11. Stomatal frequency and distribution on newly and fully expanded laminae of ACD plant 177. ACD plant 177 had low yield under drought condition in the field experiment and it died during recovery. The adaxial surface (upper Figure) had more stomata than abaxial (adaxial : abaxial = 8.51) but they were smaller and relatively irregular. The abaxial surface (bottom Figure) had less stomata but these were relatively larger, and relatively regular (Magnification was 10 × 10X)





In other species, stomatal frequency and size in leaves of eight bread wheat, one self-fertile, homozygous, dwarf rye cultivar and their newly synthesized amphiploids were determined (Singh and Sethi, 1995). All the amphiploids and their parents (wheat cultivars and rye) where hyperstomatous. The wheat and the amphiploids were significantly different in stomatal frequency. Rye cultivar showed significantly grater numbers of stomata than wheat and most of the amphiploids. A few amphiploids resembled rye for stomatal frequency in the leaf next to the last. From comparing these two programmes, anther culture in *Lm/Fa* pentaploid hybrid seems were more successful in producing variation than crossing rye and wheat cultivars. Significant positive correlation between ad- and abaxial in wheat, rye and their amphiploids (Singh and Sethi, 1995) was in contrast with non correlation between stomata number of ACD plants on leaf surfaces (Table 7.1).

The stomatal frequency of ACD plants were not similar to the *Lm/Fa* pentaploid hybrid neither on adaxial nor abaxial (Table 7.1 and as examples Figure 7.10 and 7.11). Total stomatal frequency on leaf surfaces was from a minimum of 115.7 in plant 84 (less than control species) to a maximum of 290.4 in plant 68. Plant 68 had more yield and tiller than plants 84 under drought conditions in the field but also there were plants (e.g. Nos. 219 and 193) with more stomatal frequency than plant 68 and higher yielding under drought and irrigated conditions. Although the adaxial and abaxial stomatal frequency of plant 219 (Figure 7.10) differed from plant 177 (Figure 7.11), their total stomatal frequency of (163.7 and 183.6 respectively) was almost similar. While plant 219 was the most drought tolerant and plant 177 was one of the sensitive plants (see Chapter 6).

Chi-square tests for stomatal frequency and distribution on both adaxial and abaxial leaf surfaces, total stomatal frequency on leaf surfaces and adaxial : abaxial ratio are presented in Table 7.1. Genotypes differed for all above-mentioned factors ($P > 0.01$). The source of variation of stomatal frequency on the adaxial leaf surface was the high stomatal frequency of ACD plants Nos. 68 and 6, and the lower stomatal frequency of *L. multiflorum* and *F. arundinacea* species and plant 54. The

main source of variation of stomatal frequency on the abaxial leaf surface was due to the highest frequency on plants Nos. 74 and 68 and the lower stomatal frequency of plants Nos. 177 and 38. However plant Nos. 133 and 68 which had a high stomatal frequency and plants Nos. 84, 54, 51 and *L. multiflorum* a low stomatal frequency were the source of significant variation of total stomatal frequency among these genotypes.

The stomatal frequency on the adaxial leaf surfaces (Figure 7.12) of *L. multiflorum* and *F. arundinacea* were almost equal while the *Lm/Fa* pentaploid hybrid had more stomata on this surface than either parent species. All ACD plants had more stomata on their adaxial leaf surface than the control species and 6 ACD plants had more stomata on the adaxial leaf surface than the *Lm/Fa* pentaploid hybrid. The ACD plant with the greatest stomatal frequency (No. 68) had 2.78 times more stomata than plant 54 with fewest stomata. Numbers of stomata on abaxial leaf surfaces (Figure 7.13) of most ACD plants were less than *F. arundinacea.* The hybrid had less stomata on the lower leaf surface than either *L. multiflorum* and *F. arundinacea* and most of ACD plants. Only two ACD plants had more stomata on their abaxial surface than both parents.

The total stomatal frequency (on both sides of the leaf) (Figure 7.14) of *F. arundinacea* was slightly more than *L. multiflorum* and both of them had less stomatal frequency than the *Lm/Fa* pentaploid hybrid. Most ACD plants (14 plants) had higher stomatal frequency than both *L. multiflorum* and *F. arundinacea*. The ACD plant 68 (with the maximum stomatal frequency) had 2.51 times more stomatal frequency than plant 84 (with minimum stomatal frequency). While the adaxial : abaxial ratio of leaf stomatal frequency (Figure 7.15) for *L. multiflorum* and *F. arundinacea* was less than unity, this value was greater than unity for all ACD plants (except plant 74). This ratio for ACD plant 177 was 9.05 times greater than plant 74. Figure 7.16 shows any relationship between stomatal frequency on the adaxial and abaxial leaf surfaces.

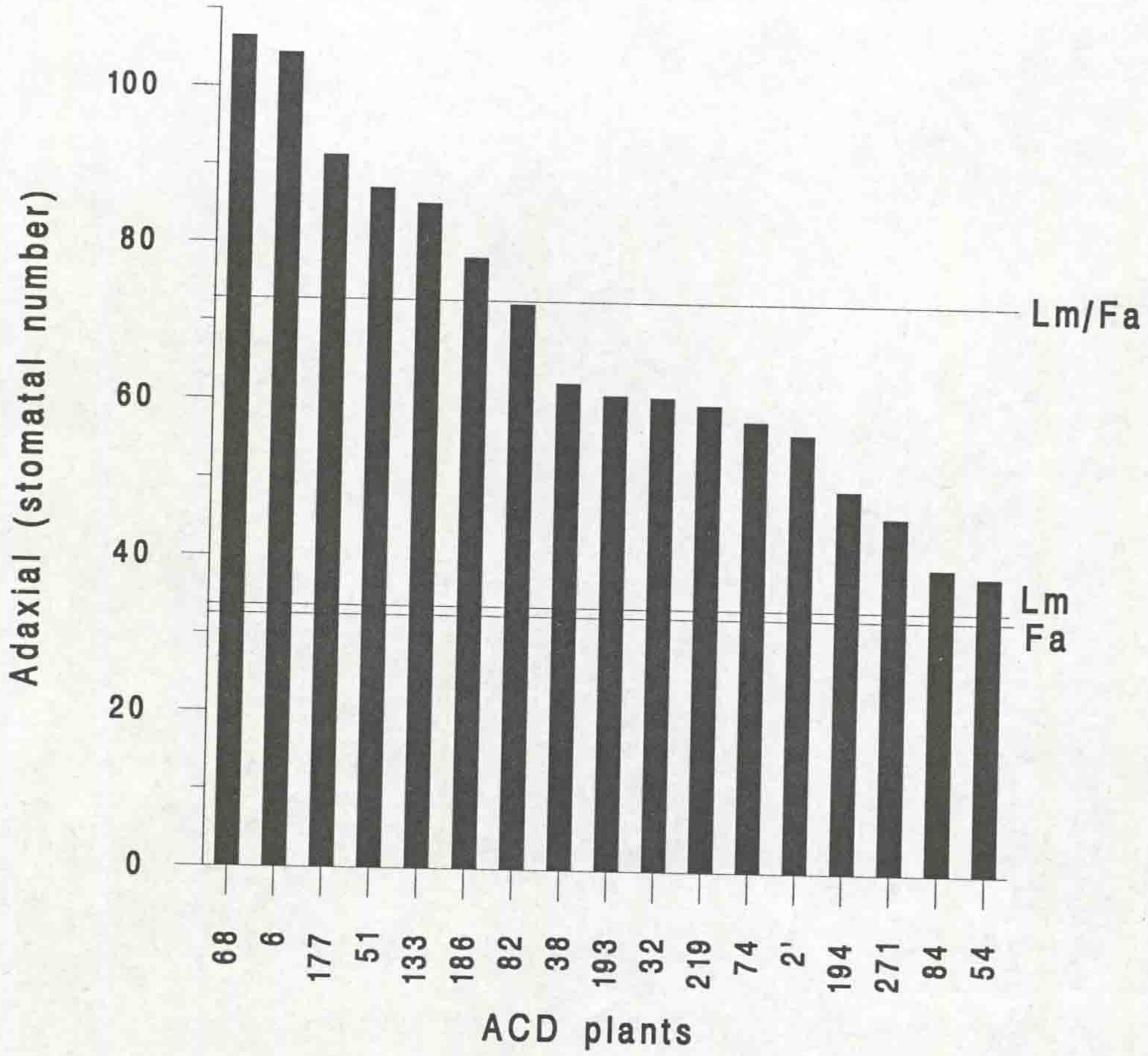


Figure 7.12. Stomatal frequency on adaxial leaf surfaces. Each point is mean (stomata/mm^2) of three microscope (10 × 10X) fields. Lm = *L. multiflorum*, Fa = *F. arundinacea*, Lm/Fa = *L. multiflorum* × *F. arundinacea* and Nos. (2'-219) = ACD plant number.

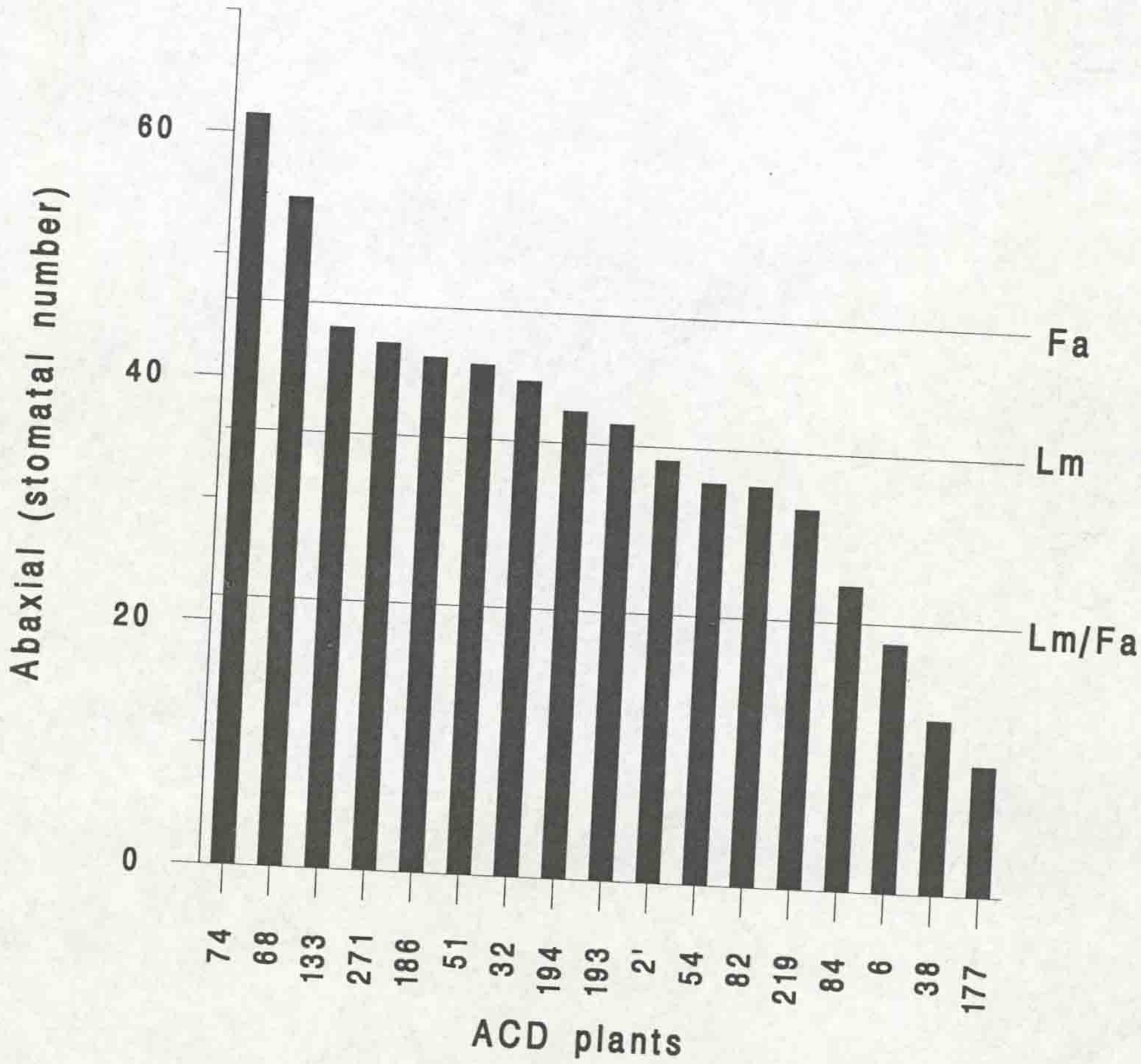


Figure 7.13. Stomatal frequency on abaxial leaf surfaces. Each points is mean (stomata/mm^2) of three microscope (10 × 10X) fields. Lm = *L. multiflorum*, Fa = *F. arundinacea*, Lm/Fa = *L. multiflorum* × *F. arundinacea* and Nos. (2'-219) = ACD plants number.

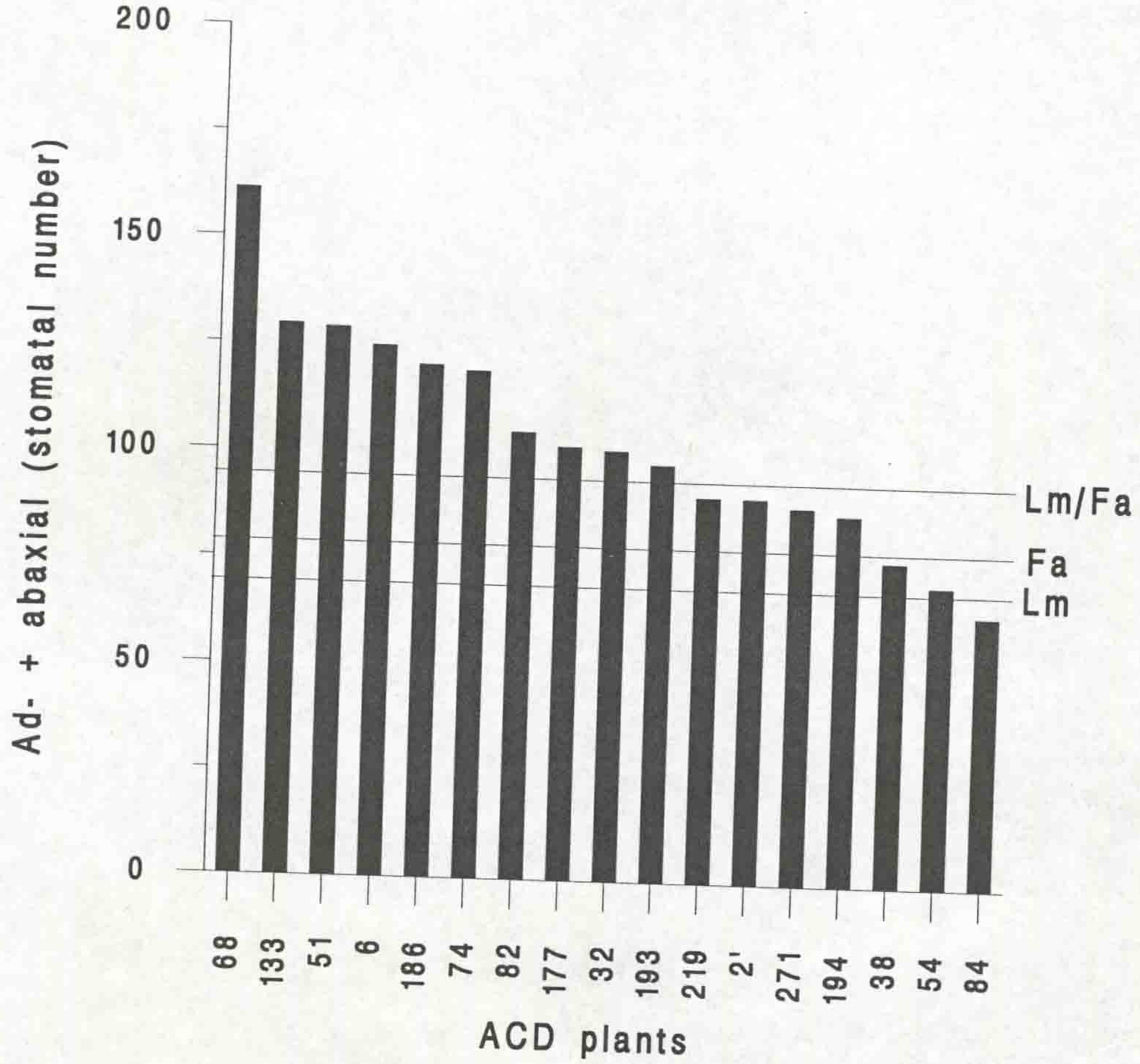


Figure 7.14. Stomatal frequency on total stomatal frequency on both leaf surfaces. Each points is mean (stomata/mm^2) of three microscope (10 × 10X) fields. Lm = *L. multiflorum*, Fa = *F. arundinacea*, Lm/Fa = *L. multiflorum* × *F. arundinacea* and Nos. (2'-219) = ACD plants number.

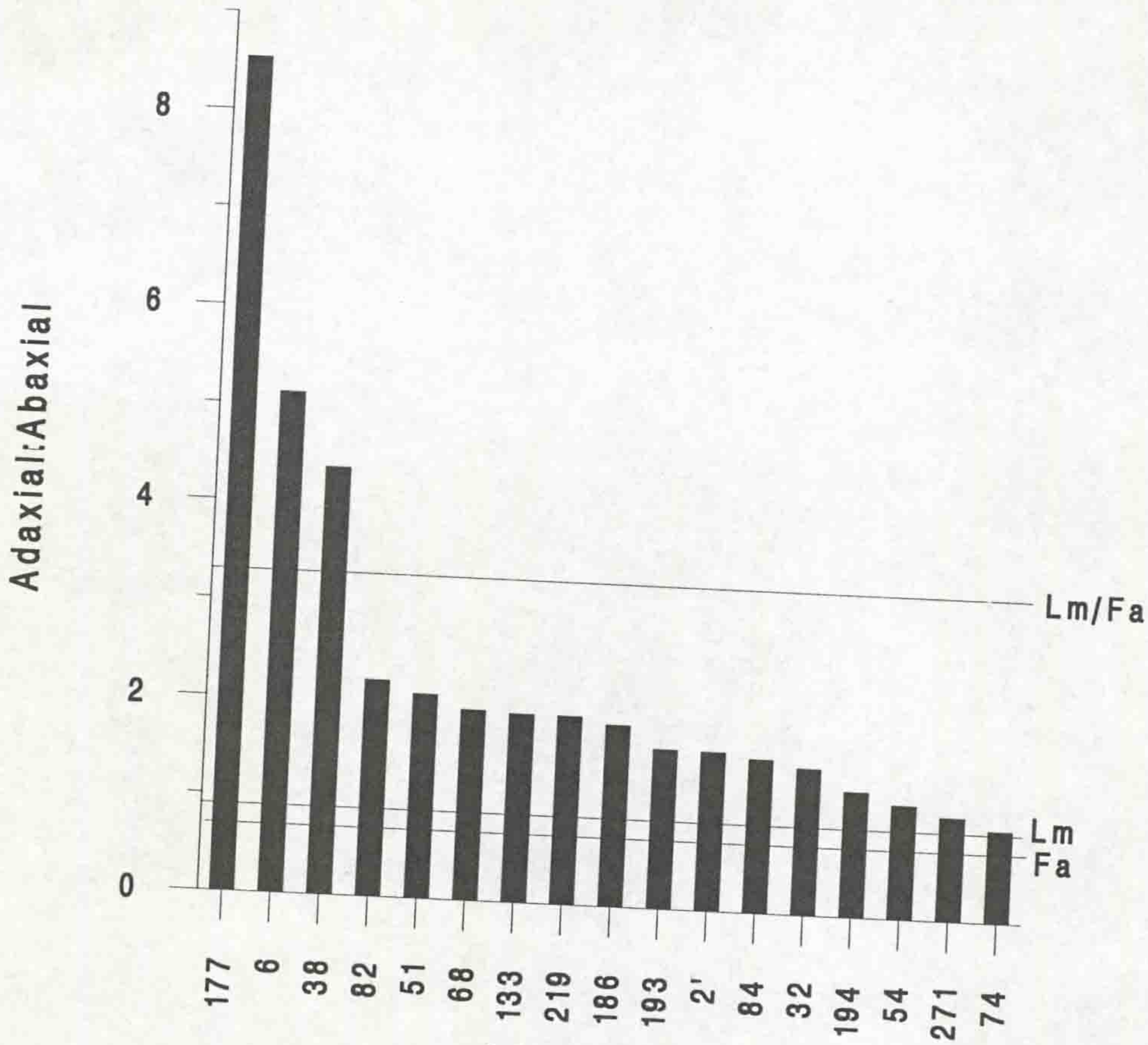


Figure 7.15. Adaxial : abaxial ratio of stomatal frequency on leaf surfaces. Each point is mean (stomata/mm^2) of three microscope (10 × 10X) fields. Lm = *L. multiflorum*, Fa = *F. arundinacea*, Lm/Fa = *L. multiflorum* × *F. arundinacea* and Nos. (2'-219) = ACD plants number.

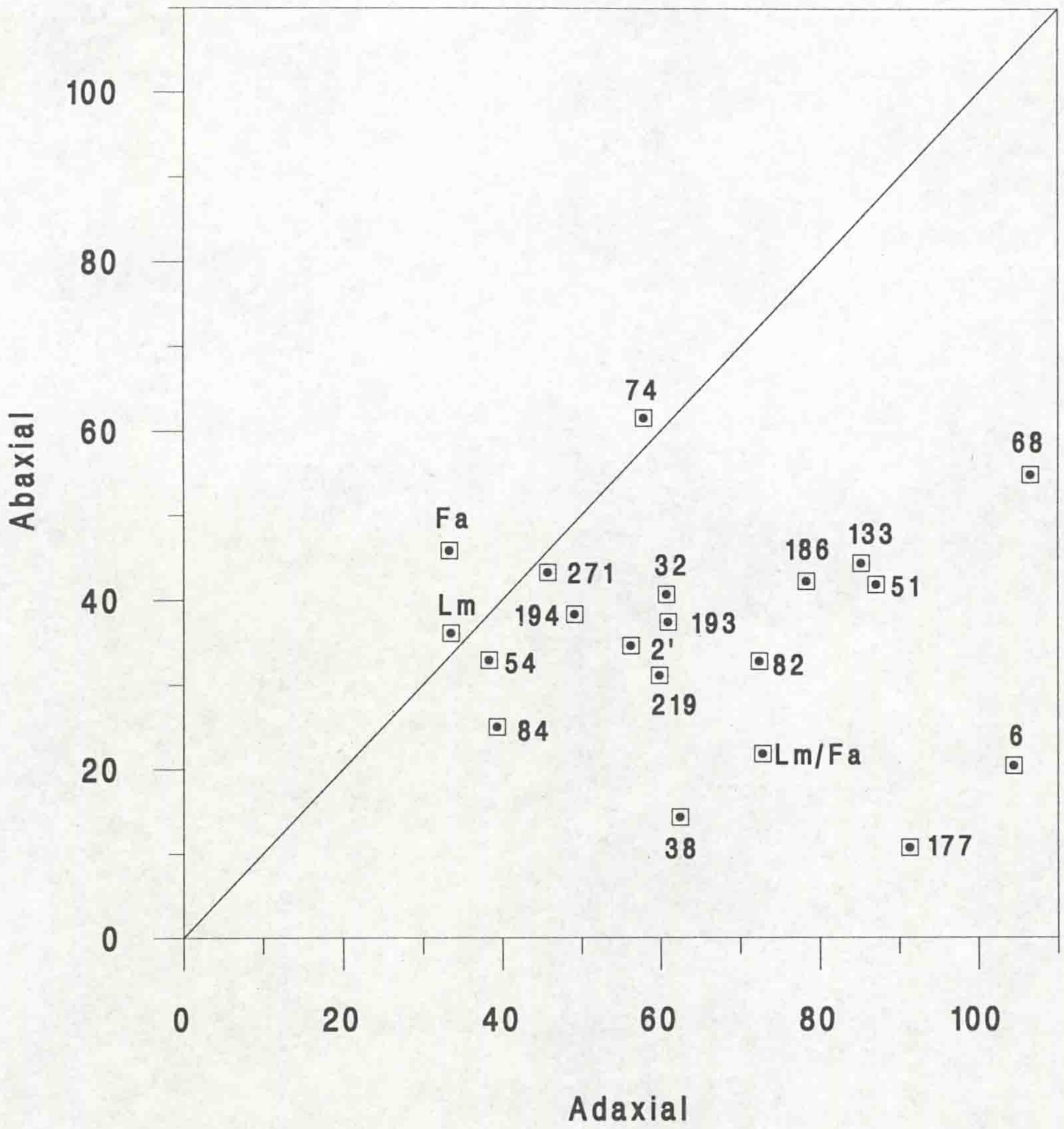


Figure 7.16. Relationship between stomatal frequency on adaxial and abaxial of leaf surfaces of ACD plants and their three control genotypes. Selected genotypes are labelled. Lm = *L. multiflorum*, Fa = *F. arundinacea*, Lm/Fa = *L. multiflorum* × *F. arundinacea* and Nos. (2'-219) = ACD plants number.

7.3.3. Relationship of stomatal frequencies and distribution with cold and drought

In some cases a degree of relationship between stomatal frequency and environmental stress had been reported. Stomatal density appeared to be the predominant character controlling the water status of Golden Delicious apple trees in the period of vegetative growth in spring (Elias, 1995). Using heat-balance stem gauges, sap flow rates were continuously measured in two pairs of soybean isolates with differing stomatal frequency (Tan and Buttery, 1995). They found more water transpiration in plants with high stomatal frequency than in plants with low stomatal frequency at high moisture level. Under low soil moisture levels, the water use rate decreased greatly for the high stomatal frequency plants. Plants with low stomatal frequency were able to maintain greater rates than with high stomatal frequency. Hattalli *et al.* (1993) observed higher stomatal frequency on the abaxial leaf surface and lower interveinal distance in higher yielding wheat genotypes. On the other hand, Jarvis and McNaughton (1986) showed that in field crops transpiration was controlled mainly by meteorological conditions, and that stomatal resistance was of secondary importance. Nevertheless, high stomatal resistance, and therefore possibly low stomatal density, is likely to conserve water in the leaves, if not in the soil.

F. arundinacea appears to express its drought resistance, at least in part, through effective control of water loss. Therefore, relationships were sought between stomatal characters and plant growth rate.

The correlation coefficients (with and without control genotypes) were calculated between (a) plant growth rate (PGR, mgd^{-1}) [at the early drought stage in the field (cut 1 to cut 2), at late drought stage (cut 2 to cut 3) and after recovery following drought (cut 3 to cut 4)], tiller production capacity during drought in the field (TPC %), survival score in the glasshouse [at the end of drought (d95d) and after recovery (d95r)] and freezing tolerance (LT_{50}), and (b) stomatal factors

(stomata on adaxial, abaxial, total stomata number on leaf surfaces and the adaxial : abaxial ratio). There were significant but weak correlations of abaxial density with PGR, and much stronger correlations of adaxial : abaxial ratio with PGR. Stomatal densities are probably very dependent on growing conditions, particularly because they combine the effects of development (determining which cells are stomata and which are "ordinary" epidermal cells) and cell extension (a high degree of cell extension will spread a given number of stomata over a larger area). The adaxial : abaxial ratio is probably a less biased estimate of stomatal frequency since both leaf surfaces are likely to be affected similarly. A high adaxial : abaxial ratio means that there are few stomata on the leaf surface that is exposed during drought when the leaves become rolled, and implies better water conservation and hence greater drought resistance. This could explain the close correlation between the adaxial : abaxial ratio of stomata and PGR.

CHAPTER EIGHT

A CYTOGENETIC ANALYSIS OF ANDROGENIC PLANTS DERIVED FROM THE *L. MULTIFLORUM* × *F. ARUNDINACEA* PENTAPLOID HYBRID

8.1. Introduction

Anther culture as a technique is now widely used in a range of grass species and with interspecific hybrids with increasing success (Andersen *et al.*, 1991). The advantages for plant breeding of producing instant genotype homozygosity by chromosome doubling anther culture derived lines has widely been reported (e.g. Sleper and Nelson, 1990).

From a cytogenetic aspect, androgenic plants represent the result of chromosome pairing, recombination, and disjunction. They therefore provide a very useful insight into chromosome behaviour at meiosis.

Successful breeding programmes aimed at introgression of alien genes from one species into another depends on adequate levels of interspecific chromosome pairing. The pentaploid hybrid between autotetraploid *L. multiflorum* (2n = 4x = 28) and *F. arundinacea* (2n = 6x = 42) fulfils this requirement, and has been used as a starting point in backcross breeding programmes (Humphreys, 1989; Humphreys and Thomas, 1993; Humphreys and Ghesquière, 1994).

Humphreys and Ghesquière (1994) anticipated that complex traits such as drought tolerance which are found in the hexaploid species *F. arundinacea* are likely governed by genes located on different genomes. They created the pentaploid hybrid described in this between *L. multiflorum* and *F. arundinacea* (2n = 5x = 35) with a single chromosome in each of the three genomes of *F. arundinacea* labelled with different homoeoalleles of the phosphoglucoisomerase (PGI/2) locus. The pentaploid hybrid was backcrossed onto diploid *Lolium* and recombinants recovered in the BC2 involving *Lolium* and each of the three *Festuca* genomes, establishing the efficacy of such a backcrossing programme for introgression of *Festuca* genes into *Lolium*.

This chapter describes segregation of *Lolium* and *Festuca* PGI/2 homoeoalleles within a population of androgenic plants derived from the pentaploid *Festulolium* hybrid. The segregation of PGI/2 homoeoalleles is considered a good

indicator of chromosome behaviour at meiosis in the pentaploid *Festulolium* hybrid, at least of one homoeologous group. A comparison is made between segregation of the PGI/2 alleles within the androgenic population and their recovery in BC1 progeny from the backcross breeding programme described above (Humphreys and Ghesquière, 1994). The BC1 population were derived by hybridizing *L. multiflorum* (2x) as female with *L. multiflorum* × *F. arundinacea* (5x) as male parent.

Two androgenic plants which display particularly high drought and freezing tolerance in combination with good agronomic characters (Chapter 5 and 6), are cytologically characterized in detail, using genomic *in situ* hybridization (GISH).

8.2. Materials and Methods

The pentaploid hybrid between *L. multiflorum* (2n = 4x = 28) × *F. arundinacea* (2n = 6x = 42) used for anther culture (see Humphreys and Ghesquière, 1994) had two homologous chromosomes 6 of *Lolium* (Lewis *et al.*, 1980) labelled at the phosphoglucoisomerase (PGI/2) locus by two alleles "*a*" and "*b*", respectively. Three homoeologous chromosomes of *F. arundinacea* carrying the PGI/2 locus, each based in a genome of the amphiploid *Festuca* species are labelled "a^+", "*c*", and "*e*", respectively.

The PGI/2 phenotype of plants, all derived from anther culture from the *L. multiflorum* × *F. arundinacea* hybrid, was determined. The electrophoretic procedures used to separate variants at the PGI/2 locus in leaf samples of different androgenic plant genotypes are described in Chapter 2.

Great care was taken to isolate and record the origin of different androgenic embryoids as they were formed to eliminate any possible risk that two or more embryoids might derive from the same microspore and have the same genotype. Only 221 androgenic plants were used for cytogenetic analysis, having an established different genotype on the basis of their chromosome number, PGI/2

phenotype, and origin from different calli.

The poor root development of some androgenic plants, accompanied by the low frequency of mitotic cell divisions, limited the possibilities for detailed cytological investigation of the entire androgenic plant population. Approximate chromosome number of androgenic plants was determined using a flow cytometer and carried out by Dr. Marie-Claire Kerlan at INRA, Lusignan, France. DNA content of 128 randomly selected anther culture derived plants was determined using cut leaf samples and a flow cytometer using the procedures described by Marie and Spencer, 1993. Additional flow cytometric readings were made using plants of known ploidy number as controls. These control plants were *F. arundinacea* (6x), *L. multiflorum* (2x) and (4x), and the *L. multiflorum* × *F. arundinacea* (5x) parent. The DNA readings of the control plants were compared with readings obtained from androgenic plants to establish the approximate chromosome number of anther culture derived plants. Accurate chromosome counts were achieved using Feulgen DNA stain and conventional cytological techniques on a 50 plant sample. The detailed chromosome study confirmed the accuracy of the cytometric readings.

Two anther culture derived plants, genotypes 193 and 219 with proven high drought and freezing tolerance (Chapter 5 and 6) were selected for detailed cytological examination using genomic *in situ* hybridization. The techniques used were mainly carried out by Drs. M. W. Humphreys and Izolda Pasakinskiene at IGER, Aberystwyth. We followed procedures described by Thomas *et al.*, (1994); Humphreys *et al.*, (1995), and Pasakinskiene *et al.*, (1996). Total genomic DNA (100 μg) of *Lm* and the two progenitors of *F. arundinacea* i.e. *Fp* and *Fg*, labelled with rhodamine-4-dUTP, was used as probe on mitotic chromosome preparations of the two anther culture derived plants. As blocking DNA, x40 probe concentration of *F. arundinacea* DNA was used with the *Lm* probe, and of *Lm* DNA with either of the *Festuca* probes. Following *in situ* hybridization, chromosome preparations were counterstained with DAPI and examined under a Leitz Epifluorescence microscope and combined DAPI-rhodamine images captured on Kodak Elite 400 film.

8.3. Results and discussion

Two hundred and twenty one green androgenic plants derived from different microspores and thus having different genotypes, were successfully established in soil. The plants had very diverse morphology with contrasting leaf size, tiller number, growth habit, plant height, and root/shoot ratios (see Chapter 4). The plants also differed widely in their physiological characteristics, with plants of different growth rate, tillering capacity, and drought and freezing tolerance (Chapter 4, 5 and 6).

The total frequency of the 5 PGI/2 homoeoalleles in the 221 androgenic plants is given in Table 8.1. The origin of the 5 PGI/2 alleles in the pentaploid hybrid used as parent, was previously described by Humphreys (1995). He demonstrated that the "a^+" and "*e*" alleles were located in different genomes of *F. arundinacea* on homoeologous *Fg*, Fg_1 chromosomes. The "*c*" PGI/2 allele labelled a third homoeologous *Festuca* chromosome in the *Fp* genome of *F. arundinacea*. The two PGI/2 homoalleles "*a*" and "*b*" labelled to homologous *Lm* chromosomes in the pentaploid *Festulolium* hybrid.

The frequency of the 5 PGI/2 alleles in the androgenic plant population differed significantly ($\chi^2_{[4]} = 15.81$, $P \leq 0.01$). There was no difference in frequency between any of the three *Festuca* alleles ($\chi^2_{[2]} = 1.61^{NS}$). The difference in frequency of the 5 PGI/2 alleles was explained by the high number of *Lm* "*a*" alleles, which occurred more frequently in the androgenic population than the other *Lolium* PGI/2 allele, the "*b*" allele ($\chi^2_{[1]} = 6.36$, $P \leq 0.05$). The *Lm* "*b*" allele was at a similar frequency to the three *Festuca* PGI/2 alleles ($\chi^2_{[3]} = 4.49^{NS}$).

Table 8.1. The frequency of PGI/2 alleles in androgenic plants, each marking chromosomes from different homoeologous groups in the parent *L. multiflorum* × *F. arundinacea* (2n = 5x = 35) hybrid (PGI/2 = $a^{+}abce$).

PGI/2 alleles					Total number	
Lolium		*Festuca*			Alleles	Plants
a	b	a^{+}	c	e		
139	100	102	97	85	523	221
(26.6%)	(19.1%)	(19.5%)	(19.55%)	(16.25%)		
Total $\chi^2 = 15.81$**						

** = $P \leq 0.01$

The PGI/2 phenotypes of the 221 androgenic plants and their frequency are given in Table 8.2. No plants carried more than 3 PGI/2 alleles. One plant was obtained with a single PGI/2 "*b*" *Lm* allele. A total of 138 plants carried 2, and 82 carried 3, PGI/2 alleles.

A *Lm* PGI/2 allele was found in 98% of the androgenic plants with 10% carrying both *Lm* "*a*" and "*b*" alleles. The recovery of the *Festuca* PGI/2 alleles was not as regular as *Lolium*. Among the 221 androgenic plants, 30% carried no *Fg* PGI/2 alleles, and 14% carried both *Fg* alleles. The *Fp* PGI/2 "*c*" allele was recovered in just under half the plants (44%).

Table 8.2. The possible and observed PGI/2 phenotypes in 221 androgenic plants derived from a *L. multiflorum* × *F. arundinacea* (5x) hybrid (PGI/2 = a^+abce).

No. of alleles	Phenotype	Frequency	Total
5	a^+abce	0	0
4	a^+abe	0	
	a^+ace	0	
	a^+bce	0	0
	abce	0	
	a^+abc	0	
3	a^+ab	18	
	a^+ac	19	
	a^+ae	13	
	a^+bc	6	
	a^+be	16	82 (37.1%)
	a^+ce	1	
	abc	3	
	abe	0	
	ace	3	
	bce	3	
2	a^+a	12	
	a^+b	15	
	a^+c	0	
	a^+e	2	
	ab	1	138 (62.4%)
	ac	43	
	ae	27	
	bc	18	
	be	19	
	ce	1	
1	a^+	0	
	b	1	1 (0.5%)
	c	0	
	e	0	
0	-	0	0

Table 8.3. Recovery and transmission of PGI/2 alleles in androgenic plants and BC1* involving the identical *L. multiflorum* × *F. arundinacea* (5x) hybrid (PGI/2 = *a*$^{+}$*abce*).*

*The BC1 were derived from *L. multiflorum* (2x) × (*L. multiflorum* × *F. arundinacea*) (5x) (Humphreys and Ghesquière, 1994).

PGI/2 alleles		Androgenic plants			BC1		
		Alleles		Total No	Alleles		Total No
		No.	%	plants	No.	%	plants
a^{+}	*Fg*	102	19.5		27	12.4	
a	*Lm*	139	26.6		52	23.9	
b	*Lm*	100	19.1	221	53	24.3	117
c	*Fp*	97	18.6		50	22.9	
e	*Fg*	85	16.2		36	16.5	
Total no. alleles		523			218		
χ^2 Comparison of PGI/2 alleles frequency in androgenic and BC1 population.					$\chi^2_{[5]} = 8.43^{NS}$		

Fg = *F. glaucescens* PGI/2 allele

Lm = *L. multiflorum* PGI/2 allele

Fp = *F. pratensis* PGI/2 allele

In Table 8.3, the frequency of the 5 PGI/2 alleles in the androgenic population was compared with their transmission via the pollen into the BC1 between *L. multiflorum* (2x) × (*L. multiflorum* × *F. arundinacea*) (5x) (described by Humphreys and Ghesquière, 1994). There was no significant difference ($\chi^2_{[5]}$ = 8.43) in the frequency of PGI/2 homoeoalleles in the androgenic *Festulolium*, and in the BC1 population. Consequently, there was no obvious selection in the BC1 against any chromosome from the PGI/2 labelled homoeologous chromosome set found in the pentaploid *Festulolium* hybrid. *Lm*, *Fp*, and Fg/Fg_1 PGI/2 alleles were transmitted to the BC1 in accordance with their frequency in androgenic plants and in viable pollen microspores.

Chromosome number of 128 randomly selected androgenic plants was determined using a flow cytometer. Root tip mitotic chromosome counts of 50 androgenic plants confirmed the accuracy of the cytometric results. The chromosome number of androgenic plants is summarised in Table 8.4. Chromosome number ranged between 14 chromosomes and >35 but the majority of plants were 14 to 21 chromosomes. There was little evidence amongst the androgenic population of spontaneous chromosome doubling, and the majority of plants were aneupolyhaploids.

Table 8.4. Chromosome number of androgenic plants derived from a *L. multiflorum* × *F. arundinacea* (5x) hybrid determined both by flow cytometry and from chromosome counts at root-tip mitosis.

Chromosome number	Number of plants	Total (%)
14	28	22
15-20	51	40
21	28	22
22-27	2	1.6
28	12	9
29-34	2	1.6
35	4	3
36+	1	0.8
Total	128	100

Two androgenic plants, genotypes 193 and 219 which combined drought and freezing tolerance with good agronomic characters such as high dry matter production, good root/shoot ratios and high tillering rates, were chosen for a detailed cytological study using GISH. Root-tip mitotic chromosome preparations of 193 and 219 probed with *Lm* DNA labelled with rhodamine-4-dUTP and with *F. arundinacea* as blocking DNA are presented in Figure 8.1. The *Lm* DNA hybridized preferentially onto *Lm* chromosomes, and to introgressed *Lm* chromosome segments on *Festuca* chromosomes. The *Lm* DNA probe partially hybridized to certain *Festuca* chromosomes but failed to hybridize with other *Festuca* chromosomes. The *Lm* genome is known to be structurally closer to *Fp* than to *Fg* (Humphreys, 1995; Pasakinskiene *et al.*, 1996) and the *Festuca* chromosomes which partially hybridized with the *Lm* probe were likely those of *Fp*. The chromosomes which showed no evidence of hybridization with the *Lm* probe were *Fg*. Some chromosome segments

with partial hybridization to the *Lm* probe were observed on otherwise unlabelled chromosomes. They were considered to be "*Fg*" chromosomes carrying "*Fp*" introgressions.

By using *Lm*, *Fp* and *Fg* DNA as probes in turn, on different mitotic chromosome preparations of the two androgenic lines (not illustrated), the chromosome composition of plants 193 and 219, could be determined. The conclusions derived from the different levels of hybridization observed between chromosomes of genotypes 193 and 219 and the *Lolium* probe (Figure 8.1), were all confirmed. In chromosomes with alien recombinant segments, chromosomes were assigned as *Lolium* or *Festuca* by the presence of either *Lm*, *Fp*, or *Fg* DNA on both sides of the centromere. Genotype 193 had 21 chromosomes; 8 *Lm*, 7 *Fp*, and 6 *Fg* chromosomes. A single *Lm* recombinant chromosome segment was observed on a *Festuca* chromosome, and 2 *Festuca* recombinants were found on *Lm* chromosomes (Figure 8.2). The *Lm* recombinant was on a *Festuca* chromosome which did not hybridize to the *Lm* DNA probe, considered to be *Fg* chromosomes. A small terminal *Fg* recombinant segment was observed on a large *Lm* chromosome. A large terminal *Fg* recombinant occupying half a chromosome arm was found on another large *Lm* chromosome.

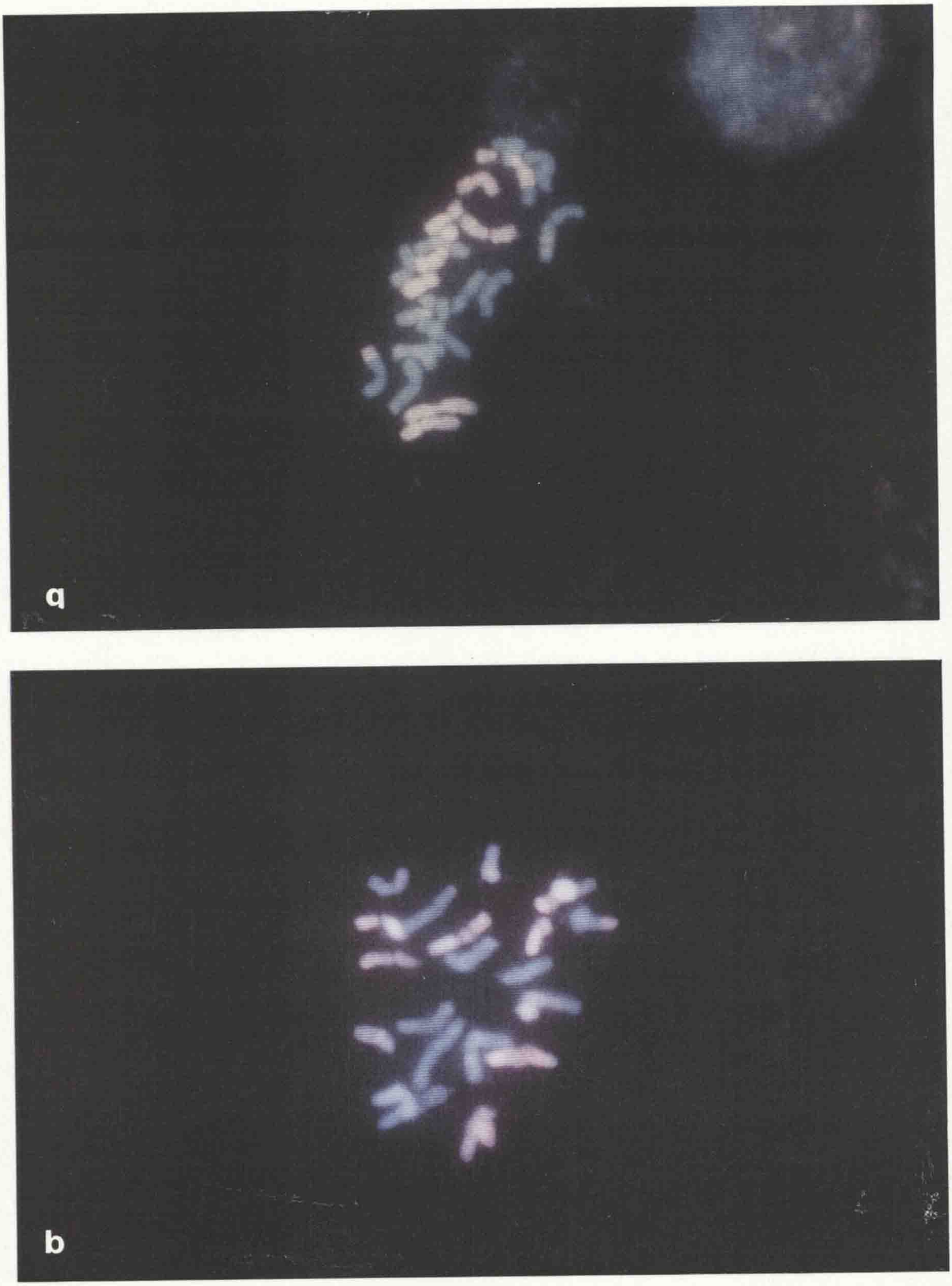


Figure 8.1. Genomic *in situ* hybridization of root tip chromosomes of anderogenic genotypes of *L. multiflorum* × *F. arundinacea* pentaploid hybrid (a) 193 and (b) 219. (Pink colour = *L. multiflorum*, blue colour = *F. glaucescens* and grey colour = *F. pratensis*.)

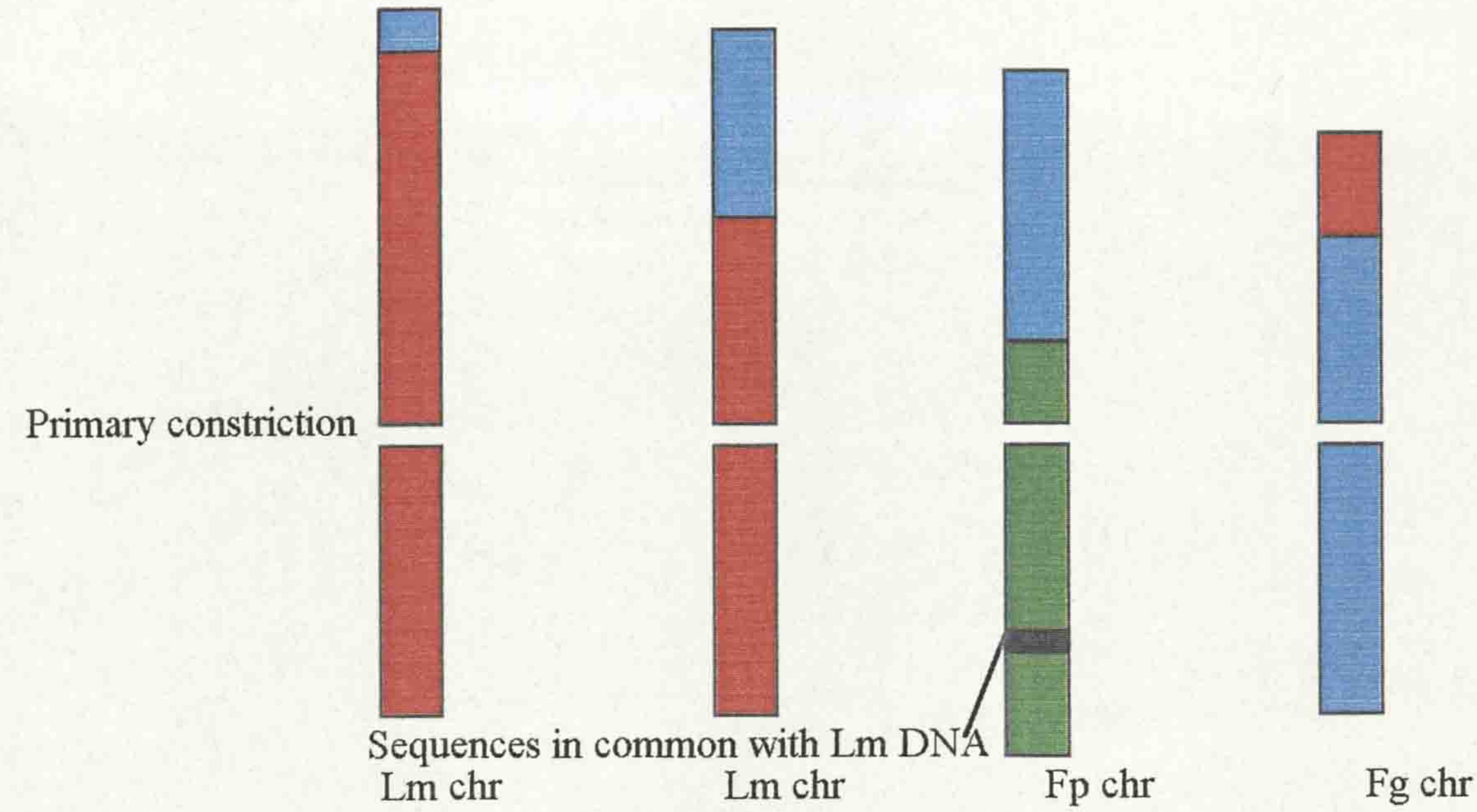
A large *Festuca* chromosome was observed which partially hybridized to the *Lm* probe but also carried a chromosome segment at its distal end, which failed to hybridize to the *Lm* DNA. This chromosome was considered to be *Fp* with a *Fg* recombinant. An intercalary band which hybridized to the *Lm* probe on the chromosome arm without the recombinant, was further evidence that this chromosome was *Fp*. A similar sequence which hybridizes to *Lm* DNA has regularly been observed at the same position on a chromosome known to be that of *Fp* (Humphreys, unpublished results).

Genotype 219 also had 21 chromosomes; 7 *Lm*, 6 *Fp*, and 8 *Fg* chromosomes. Recombinants involving all 3 genomes were clearly observed (Figure 8.2). Two of the 7 *Lm* chromosomes carried near complete *Fp* chromosome arms. One of the *Lm* chromosomes is a large satellite chromosome identified as chromosome 2 with a long arm which is almost all *Fp*.

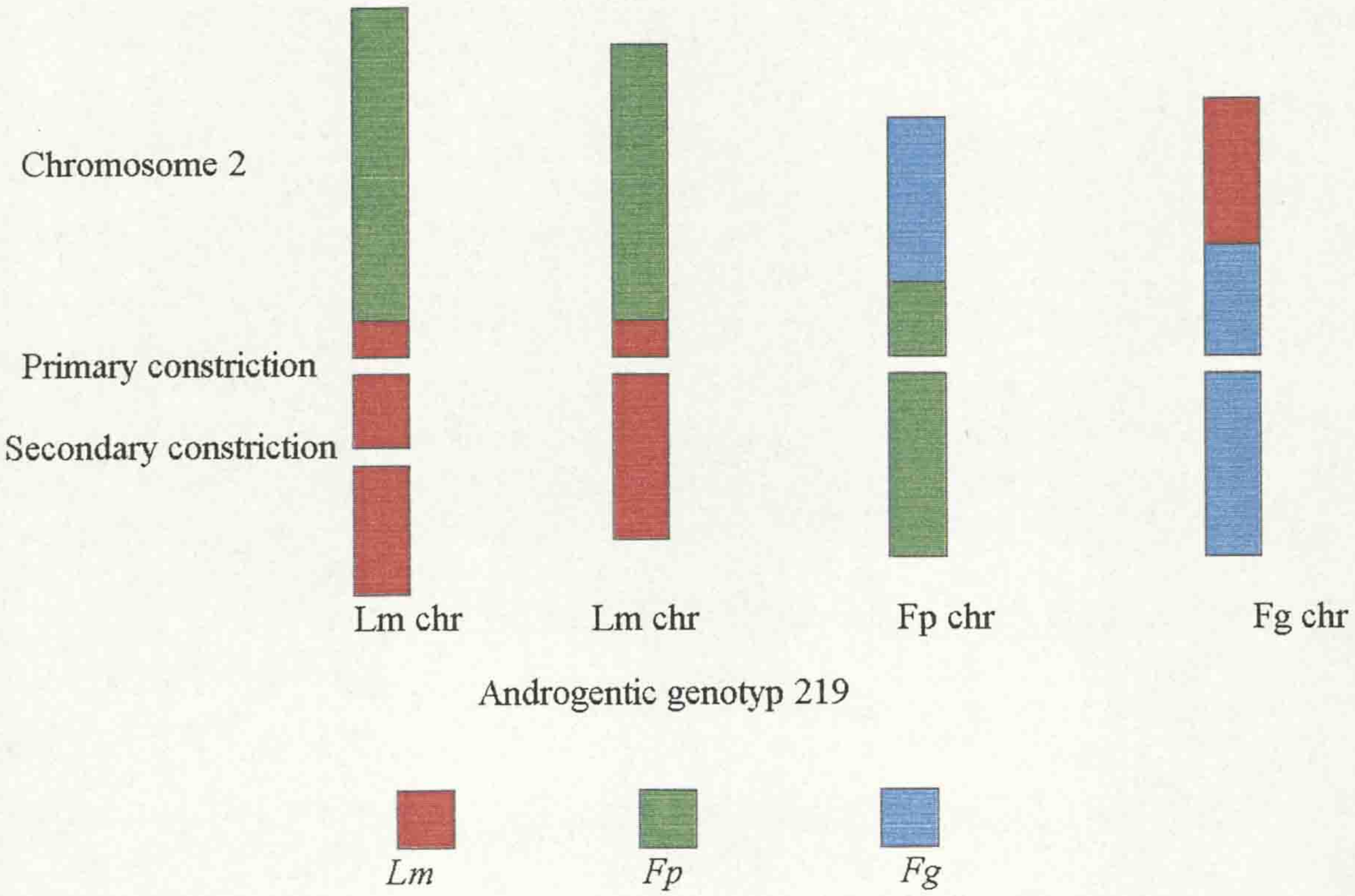
One of the 6 *Fp* chromosomes carries a distal chromosome segment which shows no signs of hybridization to the *Lm* probe and is thus considered to be a *Fg* recombinant chromosome segment.

Between the 8 *Fg* chromosomes, only one chromosome clearly carried a recombinant, a large distal *Lm* chromosome segment.

Development from microspores leads to monoploid plants and enables subsequent production of homozygous plants by chromosome doubling. This is an important step in many plant breeding programmes to improve the genetic gain during selection and achieve stability and uniformity during multiplication of selected material (e.g. Mayer *et al.*, 1995).



Androgenetic genotype 193



Androgentic genotyp 219

Figure 8.2. Diagrammatic representation of genomic *in situ* hybridization (GISH) of root tip chromosomes of androgenic genotypes of *L. multiflorum* × *F. arundinacea* pentaploid hybrid (a) 193 and (b) 219. (Green colour = *L. multiflorum*, blue colour = *F. glaucescens* and red colour = *F. pratensis*.)

CHAPTER NINE

GENERAL DISCUSSION AND CONCLUSION

Grass species have become specifically adapted to a fairly narrow range of growing conditions during the course of evolution. For this reason, an important objective in plant breeding is to produce through hybridizing grass species with contrasting adaptive qualities and growth characteristics, genotypes better adapted to selected abiotic or biotic stresses, and with improved growth characters and yield potential (Breese and Lewis, 1984). The additional modern pressures for high quality forage combined with environmentally friendly farming systems have only served to increase the pressure on plant breeders to produce versatile new cultivars.

It is widely considered that the most important grass species for use in temperate grasslands are those within the *Lolium/Festuca* complex (Thomas and Humphreys, 1991) *Phleum* species have up to now been used more widely in Northern latitudes and *Dactylis* species under drier Meditreranean growing conditions. These highly stress tolerant species lack many of the agronomic attributes of *Lolium* species and may indeed eventually be replaced in farming systems as more stress tolerant *Festulolium* cultivars are developed.

Species within the *Lolium/Festuca* complex display wide variations in adaptation to climatic stress and agronomic potential. Fortunately, there is a high degree of interfertility within the *Festuca* and *Lolium* genera and opportunities arise for combining the best attributes of both genera in a single genotype. Many natural and artificial hybrids now exist (Thomas *et al.*, 1995) and extensive collections of valuable *Festulolium* germplasm have been created (Zwierzykowski *et al.*, 1996).

A polyploid series exists within *Festuca* with polyploid species having evolved through hybridization between diploids to produce genotypes which may be better adapted to more extreme growing conditions normally than can be tolerated by the progenitor species (Humphreys *et al.*, 1993). Humphreys *et al.* (1995) have shown that the allohexaploid species *Festuca arundinacea* (*Fa*) (2n = 6x = 42) is derived from the natural hybridisation of *F. pratensis* (*Fp*) (2n = 2x = 14) and an allotetraploid species *F. glaucescens* (FgFg1) (2n = 4x = 28). The progenitors of *F. glaucescens* remain unknown. The distribution of *F. arundinacea* is wider than that

of either *F. pratensis* or *F. glaucescens.* *F. arundinacea* therefore represents an example of successful natural hybridization within one sub-section (the *Bovinae*) of the *Festuca* genus. Despite the widespread use of *F. arundinacea* as a forage crop and for amenity purposes, its generally poor forage quality and digestibility make its use for agriculture inferior to that of *Lolium*. Greater advantages will surely accrue by combining in a single genotype the complementary characters which exist between *Lolium* and *Festuca* species. The fairly close relationship between the ryegrass species and the broad-leaved fescues has enabled hybrid production and the commercial use of some *Lm* × *Fa* cultivars e.g. Kenhy in the USA (Buckner *et al.* 1977). Breese *et al.*, (1981) considered that between all *Lolium* and *Festuca* species, the combination of tall fescue (*Festuca arundinacea*) with Italian ryegrass (*Lolium multiflorum*) would offer the greatest complementation in characteristics. As such, it has received perhaps wider attention than most other interspecific grass hybrids (Terrell, 1966).

L. multiflorum with *L. perenne* are considered the most agriculturally important forage grasses for use in temperate regions (Stapledon and Davies, 1940) and as such, *Lolium* species are generally regarded as the basis of grassland improvement. The two Italian and perennial ryegrasses together account for over 80% of agricultural grass seed used in the United Kingdom (Breese, 1983). Italian ryegrass is outstanding in establishment and early spring growth, with high nutritive quality, digestibility, and water soluble carbohydrate. It provides heavy conservation crops but unfortunately is non-persistent and is thus largely used in short-term leys (Breese and Lewis, 1984 and Thomas and Humphreys, 1991).

F. arundinacea is an outbreeding hexaploid species (2n = 6x = 42) considered to be an amphidiploid, involving three basic diploid genomes (Lewis *et al.*, 1980) each with a close relationship to the *Lolium* spp. enabling intergeneric recombination in hybrids (Humphreys and Ghesquière, 1994). It has widespread importance as a forage and turf grass (Kasperbauer, 1990a). Tall fescue is one of the four major agriculturally important forage grasses grown in temperate climates. It is

however, inferior for forage quality, production and establishment compared with the ryegrasses but it is drought tolerant and winter hardy and offers good persistency (Breese and Lewis, 1984; Thomas and Humphreys, 1991).

Prior to the development of interspecific breeding programmes, seed mixtures of different species were used in an attempt to combine the best attributes of individual species within a single crop. However, considerable problems frequently arose from competition and species incompatibility in seed mixtures making sward management difficult (Thomas and Humphreys, 1991). Breese (1983) reviewed some of the problems of maintaining consistent field performance in grass mixtures.

With the discovery that fertility could be restored in sterile *Festulolium* hybrids by chromosome doubling with colchicine treatment, initial attempts at combining the attributes of the two genera in a single genotype concentrated on production of amphiploids (Lewis, 1982). In any breeding programme, whether to combine genomes or to achieve genetic transfer, the limits are initially set by the numbers of progeny that can be obtained. The crossability of the species varies markedly between different genotypes of donor species as well as between species (Breese, 1983). The success of induced allopolyploidy as a breeding technique has been limited since only triticale (wheat-rye) and tetraploid hybrid ryegrass (*L. multiflorum* × *L. perenne*) have been released as cultivars and used in farming systems (Thomas, 1993). However, polyploidy provides opportunities for producing new and novel hybrids and their derivatives that are better adapted to intensive grassland systems than existing species. Unfortunately, many of these hybrids proved to be unstable and interspecific chromosome pairing led to aneuploidy and subsequent loss of fertility and sward uniformity (Thomas *et al.*, 1994). More recently with the development of molecular markers and strategies for backcrossing (e.g. Morgan *et* al. 1988; Humphreys, 1989), the high levels of intergeneric recombination characteristics of *Festulolium* hybrids have been utilised for the introgression approach. Whether it be by amphiploidy or introgression, breeding of

interspecific hybrids has now effectively replaced attempts at achieving variation through seed mixtures.

The search for increased variation and adaptation may well extend beyond the bounds of closely related species to the use of gene combinations between distantly or unrelated taxa. Crossing barriers which exist in such cases, prevent the use of conventional breeding methods and require use of new developing technology such as genetic manipulation with transgenics (Dalton *et al.*, 1995). The development and use of somatic hybridisation (Dalton, 1993) offers opportunities for overcoming crossing barriers. For example fusion of protoplasts derived from haploid tissue cultures might be useful for obtaining somatic hybrids with desired genotypes (Kaul, 1990). Biotechnology provides a powerful means to supplement and complement the traditional methods of plant improvement by permitting access to an unlimited gene pool through the transfer of desirable genes between any two species of interest, irrespective of their evolutionary or taxonomic relationships (Vasil, 1995).

This thesis however, describes a programme which utilises relatively new tissue culture techniques to enable greater selection for and use of variation achieved by conventional hybridisation and polyploidy. Within the plant kingdom polyploidy has played a significant role in speciation and a number of cultivated species are polyploids (Thomas, 1993) for example bread-wheat, oats, cotton and tall fescue. Polyploidy has featured strongly in plant evolution as a means of conserving favoured hybrid combinations during sexual reproduction. Combining different genomes with homoeoalleles in each genome increases heterozygosity and is likely to extend adaptation (Breese *et al.*, 1981). Induced polyploidy has two major consequences: a) increase in plant size, chiefly through an increased cell size, and b) preserving genetic combinations over sexual generations through genome duplication (Breese *et al.*, 1981).

9.1. Anther culture

The use of anther culture in this programme has exploited the high levels of interspecific recombination in hybrids between forage grass species within the *Festuca-Lolium* complex (Ghesquiére *et al.*, 1991). The *L. multiflorum* × *F. arundinacea* pentaploid hybrid created by Dr. Mike W. Humphreys (1989) was highly amenable to anther culture. From the anthers of the pentaploid hybrid over 2300 plantlets were produced from which at least 221 had different genotypes and established into green plants. In this study 40 green plantlets/100 anthers were produced from the pentaploid hybrid. By comparison the general level of response was better than the best of the ryegrass clones which only produced 1-2 green plants/100 anthers (Andersen *et al.*, 1991). In a very responsive genotype of the diploid *L. perenne* (Halberg *et al.*, 1990) there were a few clones that produced 11 to 59 green plants/100 anthers (Olesen *et al.*, 1988, Andersen *et al.*, 1991). Such rapid and prolific production of polyhaploids has long been of interest to grass breeders. Such plants might be used to develop homozygous lines, an important step to improve the genetic gain during selection and to achieve genetic stability during multiplication of selected material.

Anther culture has been successful in cereals and grasses for many years (Wenzel and Foroughi-Wehr, 1984), e.g. in barley (Clapham, 1973 and Wenzel *et al.*, 1995), in *Lolium* (Clapham, 1971) and in *Lolium* × *Festuca* (Nitzsche, 1970) but its commercial exploitation has been much greater in the cereals than in the forage grasses. However, progress in androgenesis both in *Lolium* and *Festuca* species and interspecific hybrids has been made (reviewed by Andersen, *et al.*, 1991) and the procedure is now widely used.

Pretreatments of the donor plant had little effect on success in anther culture of the pentaploid hybrid but there were indications that clones maintained in an unheated glasshouse were more responsive to androgenesis than clones kept in other environments (Table 3.1). This is in agreement with Bjørnstad *et al.*

(1989) in wheat and Yang *et al.* (1992) in cauliflower. Although many factors are considered to effect anther culture such as donor plant pre-treatment and vigour, and culture conditions, plant genotype has a key role.

It is difficult to draw any conclusion as to which choice of medium is most suitable for androgenesis since demands may differ even between genotypes of one species for nutrient supplies and no general recommendation can be given (Wenzel and Foroughi-Wehr, 1984). However, it can be stated that the culture medium should include minerals, organic additives, carbohydrates and hormones. As here, numerous culture media used to regenerate haploid plants from anthers are derived from a modification of the Murashige and Skoog (1962) medium.

Success with anther culture is strongly dependent on the donor plant (Guha-Mukherjee, 1973 on rice, and Bajaj, 1976 on wheat). The factor determining high anther culture production in barley was heritable and behaved as a dominant in the F1 (Islam *et al.*, 1992). Taylor and Veilleus (1992) suggested that the androgenesis response was additive so that in a one gene model for anther culture of *Solanum phureja*, the homozygous dominant genotype would express the highest response, the heterozygote would result in an intermediate response, and the homozygous recessive, would give no response in culture. Bruins and Snijders (1995) agreed that factors affecting anther culture response and green plant regeneration in wheat were additive and that their effects were controlled by numbers of dominant alleles. Through selection between anther culture responsive hybrid clones of *Lolium*, Andersen *et al.* (1991) improved their success at anther culture.

Increasing efficiency in anther culture procedures serves no useful function without knowledge of how to exploit the use of haploids once developed. Nowadays anther culture plays an important role in plant breeding by facilitating the identification and combination of appropriate germplasm (Wenzel *et al.*, 1995). For example in wheat, by adding with a phytoxin of *Fusarium* to the anther culture medium, Fadel and Wenzel (1993) were able to select

Fusarium resistant plants. At the International Rice Research Institute (IRRI), the general objective of the anther culture programme is to improve anther culture efficiency, specifically that of indica rice cultivars, and to apply this technique in rice varietal improvement (Alejar *et al.*, 1995). In a similar way as demonstrated here in the *Festulolium* plants, they were able to select anther culture lines under field conditions for good performance in different biotic and abiotic stress conditions. Fourteen anther culture lines were used in rice breeding programmes as parents because of very good phenotypic, cold tolerance, and high yielding potential (Alejar *et al.*, 1995).

Kasperbauer (1990d) compared performance of androgenic tall fescue at three ploidy levels (3x, 6x, and 12x). He concluded that (1) the 42-chromosome (6x doubled haploid) plants had the same morphology as the 21-chromosome parent haploid plants, and (2) that the doubled haploid plants were fertile and produced abundant viable seed when grown in field plots. Thus field evaluation at the haploid stage saves on time and finance and enables selection of genotypes for chromosome doubling and subsequent production of homozygous lines.

Two examples of stress tolerance assessment used in the current study are described below. Despite concentration on variation in drought and freezing tolerance in the androgenic lines, it must be remembered that other valuable agronomic characters may also be present in the genotypes studied that simply await discovery. For example *Fa* is known to offer disease resistance such as crown rust resistance, resistance to waterlogging, and better resistance to salinity than *Lm*. Different methods have been used to evaluate plants for particular environmental stresses. Five days old seedlings of cultivars of soyabean and their somaclones regenerated by tissue culture were screened in 2.5 ppm herbicide (imazethapyt) to establish the level of herbicide tolerance (Tareghyan *et al.*, 1995). Some of the somaclones showed up to three times more tolerance than their original parents.

Assessment of salt tolerance in plant progeny was based on seedling growth after two weeks in solution culture containg NaCl. The method for tolerance assessment is based in monocotyledons upon seedling root length (Kebebew and McNeilly, 1995) and in dicotyledons on seedling shoot growth in plants such as *Medicago sativa* (Al-Khatib *et al.*, 1994a&b). The method, derived from that used for quantifying metal tolerance in numerous species (McNeilly, 1994 and Shaw, 1989), has been shown to be effective as a basis for selecting for increased salinity tolerance in seven grass species (Ashraf and McNeilly, 1986) and maize (Ashraf and McNeilly, 1989).

The androgenic *Festulolium* plants described here were evaluated in several ways to assess their potential value as novel germplasm. In progressive order, the evaluations consisted of visual examination of the newly regenerated plants, cytological examination of root tips and exposure to various stress conditions (freezing temperature in controlled environments; and field and glasshouse evaluation for drought resistance).

9.2. Selection for cold tolerance

A range of variation in freezing tolerance was expected and was found among androgenic plants produced from the highly heterozygous pentaploid hybrid. While most variation could be explained by differences in chromosome segregation and recombination, there was evidence of somaclonal variation derived from anther and callus culture which may have partly influenced response to either or both freezing and drought stress.

Since winterhardiness is an important agronomic trait in many cereal crops, most methods of selection for cold tolerance have focused on cultivar and varietal trials (Fower and Gusta, 1979: Cain and Andersen, 1980 and Robertson *et al.*, 1987). In perennial grass breeding programmes for Northern temperate

climates, improvement of winter survival has a high priority (e.g. Breese and Foster, 1970; Humphreys and Eagles, 1988; Pollock and Eagles, 1988; Thomas and James, 1993). Crosses involving compatible parents of differing cold tolerance typically yield progeny exhibiting a continuous range of hardiness between the parental extremes (Guy, 1990). Use of seedlings or tillers grown under low temperature in controlled conditions is an alternative to cold screening. A close relationships was found between winter hardiness under field conditions and cold tolerance of seedlings of mature plants of grasses, white clover and winter oats (Fuller, 1979; Eagles and Fuller, 1982).

In the *Festulolium* programme described, cold tolerance was measured by environmental simulation using an polyethylene-glycol freezing tank and expressed as the lethal temperature for 50% kill (LT_{50}). The androgenic plants were cold acclimated for two weeks at -2°C. The ability to respond to cold acclimation is a significant component of winter hardiness (Guy, 1990). It has long been known that exposure to non-optimal temperatures can increase tolerance of a previously lethal temperature (Levitt, 1980 and Pollock, 1990). Almost all temperate perennial, and many annual and biennial plants can alter their tissue and cellular freezing tolerance upon exposure to a period of low, non-freezing temperature (Levitt, 1980 and Sakai and Larcher, 1987). Cold acclimation differentiates chilling-sensitive from chilling tolerant species (Guy, 1990). It is clear that the degree to which plants are hardened prior to freezing is critical to their subsequent survival (Humphreys and Eagles, 1988). The cold hardening pretreatment prior to placing tillers of the androgenic plants on the freezing tank was therefore an important component of their assessment for freezing tolerance and procedures followed those used successfully before by Fuller and Eagles, 1978.

Conditions in the freezing tank may well be considered more harsh than those normally facing plants under field conditions. Contrary to conditions in a cold tolerance test under field conditions, in this technique the whole plant (in

this experiment the isolated tiller) was exposed to the identical low temperatures. Under field conditions there may be a snow covering to reduce levels of injury of plant tissue from low temperature. In addition, in the simulated freezing trial, roots were directly exposed to freezing damage with no support or insulation from the soil. Another difference between simulated and field conditions was that the procedures employed gave a rapid response with little chance for disease to increase the cold injury.

Intracellular ice formation and dehydration are two major stress factors involved in the freezing of tissue, and intracellular ice formation may be taken as a lethal end point (Blum, 1988). The outer tissues are those most exposed to cold injury. After exposure to low temperature, plant re-growth after freezing takes place from auxillary buds (Dr. Henry Thomas, pers. comm.).

The androgenic plants demonstrated a range of cold tolerance from more sensitive than the cold sensitive *Lm* parent to more tolerant than the cold tolerant *Fa* parent. Although Humphreys and Eagles (1988), and Eagles (1989) reported a negative correlation between agronomic objectives and winter survival, growth of the most cold tolerant androgenic plants (186, 193 and 219) significantly ($P \leq 0.01$) exceeded that in tall fescue.

9.3. Selection for drought tolerance

Traditional plant breeding programmes have relied on extensive multi-location testing of genotypes to sample production at different environments (Edmeades, 1989). Thomas (1997) stressed that the most reliable and productive forage grasses in a particular environment are those adapted, by natural selection or breeding, to slight harsher conditions than those they normally experience.

Selection may be conducted in environments where drought is the main stress factor (Thomas, 1997). Establishment of different levels of drought stress

in very dry regions is possible to achieve by controlled irrigation. In the relatively wet and unpredictable rainfall of the maritime climate of Aberystwyth, drought stress was maintained in the field by using a transparent rain out shelter. The water leakage from neighbouring land to one meter depth was drained out by a stone drain-pipe. The second drought experiment took place by growing plants in 1.0 m deep containers in the glasshouse to provide greater drought stress and to remove some of the variables in the field experiment.

Thus the androgenic plants were assessed for drought resistance under two different experiments: a) under rain-out shelter, in the field, for a 110 day drought and b) under glasshouse conditions for a 77 day drought (see chapter 6). Dry matter production, tiller production (under field conditions) and survival score (under glasshouse conditions) were used to obtain an overall judgement of levels of plant drought resistance. Under drought conditions *Fa* had, as one would expect as the drought resistant parent, higher dry matter production and survival score but produced less tillers than *Lm*, which is in agreement with all previous reports for these species (e.g. Breese, 1983; Breese and Lewis, 1984 and Thomas and Humphreys, 1991). The recovery of the parent genotypes differed under the two experiments. After recovery, dry matter production of tall fescue remained higher than Italian ryegrass under the field conditions while regrowth of Italian ryegrass after irrigation under glasshouse conditions increased and exceeded that of tall fescue. The differences in recovery of the *Lm* and *Fa* genotypes in the two experiments emphasises the complexity of assessing drought resistance in plants. However, the glasshouse experiment removed many of the field variables, provided a uniform soil and growth conditions, reduced problems of plant competition through differences in plant size, and thus might be considered a more accurate method of assessing levels of plant drought resistance. Having said this, there was considerable agreement between performance of the androgenic genotypes under the two drought treatments and I

have confidence that differences in drought resistance between androgenic genotypes are real differences.

The pentaploid hybrid showed better drought resistance than both *Lm* and *Fa* species in the field drought experiments but maintained a tiller production rate similar to *Lm*. The results in the two drought treatments are evidence of the suitability of this pentaploid hybrid genotype as a starting point in the anther culture programme aimed at producing novel germplasm for commercial exploitation which combines high growth rate of *Lm* with stress tolerance of *Fa*.

Androgenic plants demonstrated large variation in drought resistance and some genotypes died because of the drought. However survival under drought exceeded that reported by Safarnejad (1996) who found after a 30 day drought period that 94% of anther cultured alfalfa plants had died. In the *Festulolium* population, many androgenic plants were not as tolerant as *Lm* and this might at least in part be because these plants were aneupolyhaploid with gene deletions or duplications which led to loss of the natural vigour found in the hybrid parent. Of course, these plants may also lack essential genes which determine drought resistance.

However, some androgenic plants under drought conditions produced the same or even higher dry matter production in the field (11.4%) and survival score in the glasshouse (22.7%) than *Fa* and produced more tillers (25.0%) in the field than *Lm*. By comparison only 3% of the backcross population, drought stressed in a similar way under rain-out shelters by Humphreys and Thomas (1993) and derived from the same pentaploid hybrid parent, had drought resistance equivalent to that of the *Fa* parent. The high recovery of drought resistance in the androgenic population is evidence for the use of anther culture as an effective procedure for selecting this trait. Some care must be taken however in over emphasising the value of androgenesis over conventional breeding procedures. Drought resistant genotypes in the backcross population described by Humphreys and Thomas maintained forage quality similar to *Lm*. No measurements were

made of the digestibility and forage quality characters of drought resistant androgenic plants, and some genotypes certainly include deleterious *Fa* characters. However, the results obtained from the androgenic population are very encouraging, and genotypes have been identified with complementary characters from both parent species and it seems likely that entirely novel gene combinations, which may include recessive alleles have been revealed.

Some androgenic plants respond differently from their parents under stress from drought. Italian ryegrass has an inherently high growth rate, and produces its herbage before drought becomes severe and is more profligate with water in the early stages of drought (Thomas, 1986). Italian ryegrass is also less able to explore for water at depth (Garwood and Sinclair, 1979), reflecting a low root:shoot ratio. Consequently, it has a low water uptake, and normally dies by the end of drought using the procedures employed here (Thomas, 1994). Tall fescue delays dehydration because it has deep roots and can roll its leaves. Many root characters affect water uptake, and these include total root mass, vertical profile distribution, maximum depth explored, branching, root diameter, root hairs, and possibly good infection by symbiotic mycorrhizae (Thomas, 1997). No measurements were taken of root morphology in the androgenic population, but some drought resistant genotypes e.g. 219 and 193 had very long well developed root systems which surely contributed to their surival under conditions of water defecit.

Plants like 360 produce large amounts of dry matter herbage before and during drought but demonstrated very poor regrowth in recovery following regular irrigation at the end of the drought treatment. ACD plants like 360 behave more like drought susceptible Italian ryegrass than the fescue parent and the morphological characters in the field (flowering habit, leaf characters, erect stems etc.), confirmed this opinion. Some other androgenic plants demonstrated tall fescue habit, e.g. 133 which had leaves, more course even than tall fescue.

9.4. Physiological characters

Androgenic *Lm* × *Fa* plants exhibit the wide variation in plant height, leaf size and shape, flowering, and growth rate in field under non-stress conditions. All the androgenic plants were inferior to the pentaploid hybrid with regard to plant height, leaf size and dry matter yield. In addition, some androgenic plants were smaller than both the *Lm* and *Fa* genotypes used as controls and others were intermediate in size. No androgenic plants produced more tillers, or had height and growth rate in excess of the *Lm* parent, although some androgenic plants had longer and/or wider leaves than *Lm*.

As one would expect, most androgenic plants had better performance in the irrigated than in the droughted treatments in both drought experiments. Some androgenic plants had similar growth response to the parental plants under both drought treatments. Boyer (1996) in a review of drought tolerance in plants concluded that it was the physiological attributes of a species that gave a superior yield of crop grain under water limited conditions. Blum *et al.* (1989) explored these attributes in sorghum where various genotypes were grown with little or no rain so they were forced to use stored soil water.

Some androgenic plants unexpectedly showed better yields (Nos. 335 and 360) and better tiller production under drought than irrigated conditions, while plants Nos. 22 and 193 had a better survival score. Some plants which had good survival score under drought, died under irrigated conditions in the glasshouse. During the summer of 1995 it was very hot, especially in the glasshouse (+40^{o}C) and plants under these conditions must have experienced considerable heat shock. Plants under irrigated conditions may be less tolerant to heat shock than their drought stressed clonal counterparts and this finding is in accordance with recent evidence obtained in trials under similar conditions in the South of France (Dr. Henry Thomas, pers. comm.). An imbalance between photosynthesis and respiration is one of the main causes of the deleterious effects of high

temperature (Taiz and Zeiger, 1991). These deleterious effects could have had a greater effect on fast growing individuals with high shoot:root ratio; which have a high transpiration rate and reduced water uptake. Levitt (1974) pointed out that at high temperature growth may stop due to the increased rate of water loss, leading to loss of turgor.

Seven androgenic plants which apparently failed to survive the drought, in fact recovered, and produced some green leaves after irrigation. One effect of drought is, normally, to encourage root growth at the expense of shoot, i.e. the root:shoot ratio increases (Dr. Henry Thomas, pers. comm.). Observations on some androgenic plants (e.g. No. 22) at the end of drought showed a high concentration of sugar-like contents in plant tissue. Although the findings require more detailed investigation, the drought stressed tissues of plants like 22, would appear to contain high carbohydrate levels which may be reallocated in the plant as a form of osmoregulator in response to drought stress. If the interpratation is correct, then this response to drought stress in these androgenic plants, regulated presumably by alternative metabolic pathways to those in other genotypes, is distinctly different to that of the *Fa* parent which did not respond to water defecit in a similar manner.

Bewley (1979) reported the critical role of cell membrane stability under conditions of moisture stress as a major component of drought tolerance. Cell membrane damage (CMD) of desiccated leaf tissue differed between androgenic plants. However the percentage of CMD did not correlate well with other characters measured under environmental stresses i.e. cold and drought. CMD was measured on mature leaf tissue, which the plants might sacrifice during severe drought in order to improve survival. It may be that there is no correlation between mature and growing tissue, and it may be better for future work that CMD is evaluated on dissected meristems.

The two parent species had almost identical stomatal frequency on the adaxial leaf surfaces, but *Fa* had higher stomatal frequency on the abaxial surface

than *Lm*. Italian ryegrass was almost completely amphistomatous, with approximately equal numbers of stomata on the ab- and adaxial leaf surfaces. Tall fescue was hypostomatous, having more stomata on the abaxial leaf surface. By contrast, the pentaploid hybrid was strongly hyperstomatous, having three times as many stomata on the adaxial leaf surface as on the abaxial. The androgenic plants exhibited even greater diversity in stomatal frequency and distribution. Adaxial stomatal frequency ranged from 69 to 192, or 53% to 146% of that in the pentaploid hybrid from which they were derived. Abaxial stomatal frequency ranged from 19 to 111, or 49% to 282% of the pentaploid hybrid values. Stomatal distributions varied from amphistomatous (in plant No. 74) to strongly hyperstomatous (in plant No. 177, Figure 7.11).

In other species, stomatal frequency and size in leaves of eight bread wheats, one self-fertile homozygous dwarf rye cultivar, and their newly synthesized amphiploid hybrids, was determined (Singh and Sethi, 1995). All the amphiploids and their parents (wheat cultivars and rye) where hyperstomatous. The wheat and the amphiploids showed significantly different stomatal frequencies. The rye cultivar showed a significantly greater number of stomata than wheat and most of the amphiploids. A few amphiploids resembled rye for stomatal frequency in the penultimate leaf. I do not know whether variation in stomatal distribution on adaxial and abaxial leaf surfaces of amphiploids created between *L. multiflorum* and *F. arundinacea* shows as little variation as that found between the wheat rye amphiploids described above. However, I can say that in the androgenic population there exists a wide range of contrasting stomatal distribution which further serves to illustrate the extent of variation that is displayed through anther culture.

It is believed that *F. arundinacea* expresses its drought resistance, at least in part, through effective control of water loss (Thomas *et al.*, 1995). It is reasonable to consider whether difference in stomatal distribution on leaves of *Lm* and *Fa* might affect water conductance under drought stress and whether

stomatal distribution might be correlated with drought resistance in the androgenic population. Therefore, relationships were sought between stomatal characters and plant growth rate under water defecit. Unfortunately, there was only weak correlation between stomatal distribution and performance under drought. Drought resistance is a very complex character, and it might be simplistic to expect it to correlate with a single character such as stomatal frequency. Such results only serve to emphasize that there is little known about the number of loci and their function in affecting resistance to environmental stresses (Hoffmann and Parsons, 1991). Secondly the frequency of stomata may indicate maximum or potential leaf water conductance, but it is not a good measure of the conductance of a leaf under stress. This is because frequency gives does not give us a value of stomatal opening and closing, the main factor controlling transpiration. Thirdly, water loss may also be reduced by leaf rolling, which was observed in the drought treatments, but was not measured. In future studies, measurements of leaf rolling and of cuticular resistance to transpiration should be under taken.

It was encouraging to observe that the process of producing plants by anther culture generated genotypes with a wide range of expression of physiological characters and drought resistance. This should enable us to identify individual components of traits that contribute to drought resistance, without their being masked by other components. Although the general lack of correlation between traits and performance does not assist a physiological interpretation of much of the data, it does have one important and positive contribution: it means that I can dissect complex traits such as drought resistance into its different components and thus more clearly assess the contribution each part plays in achieving overall stress tolerance. The results will be used for physiological and genetic studies of the basis of stress tolerance in the future, at a more intensive level.

Some androgenic plants displayed coacclimation to different abiotic stresses e.g. 193, 219 and 22 were drought resistant, and cold tolerant. They also offer other useful agronomic characters with high yield, high tiller production, and deep rooting. Selection for increased resistance to one stress appeared to increase resistance to other stresses which accorded with the view of Hoffmann and Parsons (1991).

9.5. Cytogenetic analysis

Anther culture is a valuable technique for producing novel chromosome combinations, which not only have intrinsic interest but can serve as starting points for alien introgressions or as breeding material in their own right.

Development from microspores leads to monoploid plants and enables subsequent production of homozygous plants by chromosome doubling. This is an important step in many plant breeding programmes to improve the genetic gain during selection and achieve stability and uniformity during multiplication of selected material (e.g. Mayer *et al.*, 1995).

Microspores as products of meiosis represent a vast array of genetic variation. Through androgenesis, novel characters governed by gene combinations including recessive alleles, otherwise only rarely or never observed, may be revealed and selected. Very responsive genotypes to androgenesis such as the pentaploid hybrid described, reveal much of the potential genotypic and phenotypic variation that can be generated through meiosis.

The cytogenetic analysis (chromosome counting, flow cytometry and *in situ* hybridization) and isozyme analysis demonstrated that the majority of the androgenic plants were aneupolyhaploid. Following the chromosome doubling and stabilising of selected androgenic plants they will prove a new valuable

resource to the plant breeder. One example of successful stabilisation of chromosome doubled haploid lines are those derived from hexaploid triticale × bread wheat which remained stable for over 11 generations (Wang *et al.*, 1993).

The gene(s) controlling chromosome pairing in *Fa* are inactive in the hemizygous state (Jauhar, 1975). The hexaploid fescue species is derived from two species *F. pratensis* (*Fp*) (2x) and *F. glaucescens* ($FgFg_1$) (4x) (Xu *et al.*, 1995; Humphreys *et al.*, 1995) and the pairing control gene(s) are considered to be located on one of the *Fa* genomes (Ghesquière *et al.*, 1991). The breakdown of the chromosome pairing control mechanism of *Fa* when hemizygous, leads to considerable chromosome pairing in F_1 *Lm* × *Fa* hybrids and the formation of interspecific chromosome associations (Thomas *et al.*, 1983). Despite high levels of homoeologous chromosome pairing in pentaploid *Lm* × *Fa* hybrids (Kleijer, 1984; Morgan *et al.*, 1988; Humphreys, 1989), certain chromosome pairing preferences might be anticipated. The two homologous *Lm* genomes would be expected to pair preferentially and indeed meiotic chromosome studies support this conclusion (Kleijer, 1984; Morgan *et al.*, 1988; and Humphreys, 1989).

Genetic recombination (Humphreys and Ghesquière, 1994), and *in situ* hybridization studies here and elsewhere (e.g. Pasakinskiene *et al.*, 1996), indicate that *Lm* has closer homology with *Fp* than *Fa*. It follows that more chromosome associations in the pentaploid hybrid occur between *Lm* and *Fp* than between *Lm* and *Fa* (Humphreys and Ghesquière (1994). The three *Festuca* genomes are homoeologous, and chromosome associations between *Fp* and *Fa* would also be expected when the pairing control mechanism becomes ineffective. However, clear structural differences exist between the two *Festuca* species since their chromosomes can readily be distinguished by GISH (Humphreys *et al.*, 1995).

The cytogenetic analyses of the androgenic aneupolyhaploid plants described here provides clear insights as to the accuracy of predicted chromosome behaviour at meiosis. The presence of a *Lm* PGI/2 allele in almost

every anther culture derived plant provides strong evidence for preferential chromosome pairing and regular disjunction between the two genomes of the pentaploid hybrid.

This adds to evidence from backcross breeding programmes (Morgan *et al.*, 1988; Humphreys and Ghesquière, 1994) that each gamete in the pentaploid hybrid contains a complete *Lm* genome. Ten percent of the androgenic plants carried both *Lm* chromosomes (labelled "*a*" and "*b*" at the PGI/2 locus). The number of plants with both *Lm* alleles is an indication of the frequency of intergeneric chromosome pairing and *Lm* × *Fa* multivalents. The near absence of plants with no *Lm* PGI/2 alleles is evidence that a complete *Lm* genome is generally required to provide gamete viability. It is possible that the two homologous *Lm* genomes in the pentaploid hybrid convey a certain genetic stability to enable regeneration from *in vitro* culture. Previous research from *in vitro* culture of the *Lm* × *Fa* (5x) hybrid indicated greater chromosome stability for the *Lm* genome in culture over that of the two *Festuca* species (Humphreys and Dalton, 1992).

Thirty percent of androgenic plants carry no, and 14% carry both, *Fa* labelled chromosomes. The frequent presence of androgenic plants with no *Fa* PGI/2 labelled chromosomes is evidence that a complete *Fa* genome is not essential for gamete viability.

A comparison was made between the segregation and recovery of the five PGI/2 alleles in androgenic plants with their transmission into the BC1 between *L. multiflorum* (2x) × (*Lm* × *Fa*) (5x) (see Humphreys and Ghesquière, 1994). A lower than expected recovery of a PGI/2 allele in the BC1 would be evidence of gametophytic selection through pollen competition or zygotic abortion which can occur in backcross breeding programmes and preclude recovery of certain gene combinations (Humphreys and Thorogood, 1993).

Humphreys and Ghesquière (1994) reported equal transmission of the two *Lm* PGI/2 alleles which was evidence for regular chromosome pairing between

the *Lm* genomes. It was suprising that amongst the androgenic plants, the *Lm* chromosome labelled with the "*a*" PGI/2 allele was more frequently observed than the *Lm* chromosome carrying the "*b*" allele. It is possible that certain gene combinations on the "*a*" labelled chromosome provide a selection advantage in culture for androgenesis. The transmission of the two *Lm* PGI/2 alleles into the BC1 population at equal frequency is evidence that there is no selection against *Lm* carrying the "*b*" PGI/2 allele in the pollen.

The GISH chromosome study of two genotypes selected for extreme drought and freezing tolerance proves that chromosomes from *Lm*, *Fp*, and *Fa*/Fg1 genomes are present and can be distinguished. With *Lm* DNA as probe, chromosomes of *Lm* are readily distinguished. Chromosomes of *Fp* are identified since they share some common repetitive gene sequences with the *Lm* DNA probe which leads to low levels of hybridization with *Lm* DNA. In genotype 219, a large *Fp* chromosome recombinant was observed which occupies nearly the entire length of the long arm of chromosome 2 of *Lm*. This chromosome arm of *Fp* is known to carry genes for drought resistance (Humphreys and Pasakinskiene, 1996). It is considered likely that these *Fp* genes have contributed to the extreme drought tolerance observed in genotype 219 (Chapter 6).

The *Fa* chromosomes can be identified since they are not hybridized by the *Lm* DNA probe. The ability to distinguish all three genomes using *Lm* as probe facilitates a rapid cytological analysis of hybrids involving these species.

In addition to being able to discerne chromosomes of the *Lolium* and *Festuca* species, reciprocal recombinants involving all three genomes were also observed. In the two plants studied, recombinants between *Lm* and *Fa* were approximately equal numerous than between *Lm* and *Fp*. Since Humphreys and Ghesquière (1994) were unable to observe any *Lm-Fa* recombinants among the BC1 in their breeding programme, I can only conclude that gametes carrying *Lm* - *Fa* recombinants may face severe selection pressure or following fertilization,

possible zygotic lethality. It may be that certain gene combinations resulting from *Lm-Fa* chromosome recombination recovered from anther culture are never transmitted by conventional breeding methods.

Plants 193 and 219 show coacclimation to both drought and freezing stress and considerable imbalance between the *Lm*, *Fp* and *Fa*/Fg_1 genomes. Pollen with genomic imbalance such as that found in Plants 193 and 219 may face severe selection pressure from euploid pollen and fail to fertilize (Khush, 1973). However, since there was no significant difference in frequency of *Lm*, *Fp*, and *Fa*/Fg_1 PGI/2 alleles in androgenic plants and the BC1, it is unlikely that any severe selection pressure exists which would prevent normal transmission of *Festuca* chromosomes in backcross breeding programmes with *Lolium*.

Ideally for anther culture, parent species should be chosen with high levels of chromosome pairing and recombination to maximise variation in gene composition and expression. Chromosome pairing control gene(s) are inactive in the *Festulolium* pentaploid hybrid (Jauhar, 1975), which leads to high levels of chromosome recombination and gametes (and androgenic plants) with diverse gene and chromosome constitution.

One main advantage that accrues from anther culture arises from having genes in the hemizygous state to maximize gene expression including epistasis and the ability to select for recessive alleles relevant to a specific trait.

In order to utilise the variation obtained by anther culture, it will be necessary to restore fertility to selected androgenic plants and incorporate them in breeding programmes. By chromosome doubling, preferential chromosome pairing between homologous chromosome partners will be encouraged. The selection for the pairing control gene(s) found in one of the *Fa* genomes will further encourage preferential bivalent pairing.

9.6. Conclusions

The following can be concluded from this study:

I. An anther culture method was developed for microspores of *L. multiflorum* × *F. arundinacea* pentaploid hybrid.

II. A large number of androgenic plants were produced.

III. A number of cold tolerant and drought resistant aneupolyhaploid genotypes were selected and some showed higher levels of cold and drought tolerance than the stress tolerant *F. arundinacea* parent.

IV. The relative contribution of each parental species of the *L. multiflorum* × *F. arundinacea* pentaploid hybrid and also the progenitors of *F. arundinacea*, i.e. *F. pratensis* and *F. glaucescens*, to the androgenic plants was confirmed by isoenzyme and *in situ* hybridization techniques.

V. Some recombinants were found which had not been observed previously by conventional plant breeding.

9.7. Future work

Androgenic derivatives of a *L. multiflorum* × *F. arundinacea* pentaploid hybrid reveal the high degree of variation provided by meiosis achieved through chromosome segregation and recombination between *L. multiflorum* and the genomes of *F. arundinacea* i.e. *F. glaucencens* and *F. pratensis* (Figure 8.2). Gene combinations can be selected with high tolerance to environmental stresses, combined with good quality fodder production. It will now be essential to find ways of exploiting the germplasm created by this androgenesis programme for agricultural use and to seek the suitability of selected genotypes in the development of new cultivars. Some of the gene combinations created are

unique and should fertility and stability be achieved, it is possible that in future a completely new *Festulolium* species might be created in a similar manner to the development of *Triticale*.

Possible future developments will require:

I) Doubling selected polyhaploid genotypes in order to produce homozygous lines to develop as new genotypes.

II) If a method of selecting desired gene combinations can be devised, using for example marker assisted selection (MAS), then the doubled-polyhaploid might be backcrossed onto either *Lolium* or *Festuca* parent species to produce introgression lines.

III) An intercrossing programme between doubled-polyhaploids selected for the same traits and thus hopefully carrying the same relevant gene combinations in order to produce stable homozygous populations for that trait. Such a population, if stability and fertility can be maintained could become the basis of a novel future forage crop.

IV) Selecting the gene or genes of *F. glaucescens* that control chromosome pairing (Jauhar, 1975) which are inactive in the hemizygous state. By doubling genotypes carrying the pairing gene, homozygous lines for the gene(s) will be produced. The presence of these gene(s) in the euploid state should contribute significantly towards achieving regular homologous chromosome pairing and long-term amphiploid stability.

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APPENDICES

I. Appendices of chapter 3

Appendix 3.1: Analysis of variance of the effect of environment and fertilizer treatments on anther culture productions, responded anthers from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	4	2558.56	639.64	1.97	0.1421
Error	18	5837.49	324.31	-	-
Total	22	8396.05	-	-	-

Appendix 3.2: Analysis of variance of the effect of environment and fertilizer treatments on anther culture productions, number of calluses and sub-calluses developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	4	26809.72	6702.43	0.66	0.6281
Error	18	182990.84	10166.16	-	-
Total	22	209800.55	-	-	-

Appendix 3.3: Analysis of variance of the effect of environment and fertilizer treatments on anther culture productions, total ACD plantlets developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	4	42478.21	10619.55	2.41+	0.0871
Error	18	79266.18	4403.68	-	-
Total	22	121744.38	-	-	-

Appendix 3.4: Analysis of variance of the effect of environment and fertilizer treatments on anther culture productions, albino plantlets developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	4	23744.16	5936.04	2.31	0.0969
Error	18	46167.65	2564.87	-	-
Total	22	69911.81	-	-	-

Appendix 3.5: Analysis of variance of the effect of environment and fertilizer treatments on anther culture productions, total green ACD plantlets developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	4	6726.61	1681.65	1.22	0.3376
Error	18	24840.55	1380.03	-	-
Total	22	31567.16	-	-	-

Appendix 3.6: Analysis of variance of the effect of environment and fertilizer treatments on anther culture production, green ACD plantlets transferred into sterile soil from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	4	1551.30	387.83	0.90	0.4851
Error	18	7765.66	431.43	-	-
Total	22	9316.96	-	-	-

Appendix 3.7: Analysis of variance of the effect of environment and fertilizer treatments on anther culture productions, established ACD lines in the soil from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	4	617.63	154.41	0.52	0.7250
Error	18	5387.65	299.31	-	-
Total	22	6005.28	-	-	-

Appendix 3.8: Analysis of variance of the effect of height of the tiller of panicle (cm) on anther culture productions, responded anthers from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	2	2565.82	1282.91	6.71	0.0077
Error	16	3059.81	191.24	-	-
Total	18	5625.63	-	-	-

Appendix 3.9: Analysis of variance of the effect of height of the tiller of panicle (cm) on anther culture productions, calluses and sub-calluses developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	2	29058.62	14529.31	2.27	0.1354
Error	16	102337.74	6396.11	-	-
Total	18	131396.36	-	-	-

Appendix 3.10: Analysis of variance of the effect of height of the tiller of panicle (cm) on anther culture productions, total ACD plantlets developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	2	16311.21	8144.61	1.55	0.2423
Error	16	84139.27	5258.71	-	-
Total	18	100450.48	-	-	-

Appendix 3.11: Analysis of variance of the effect of height of the tiller of panicle (cm) on anther culture productions, albino plantlets developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	2	2855.96	1427.98	0.37	0.6942
Error	16	61190.62	3824.41	-	-
Total	18	64046.58	-	-	-

Appendix 3.12: Analysis of variance of the effect of height of the tiller of panicle (cm) on anther culture productions, total green plantlets developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	2	13385.81	6692.91	8.10	0.0037
Error	16	13220.15	826.26	-	-
Total	18	26605.96	-	-	-

Appendix 3.13: Analysis of variance of the effect of height of the tiller of panicle (cm) on anther culture productions, green plantlets transferred into sterile soil from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	2	3712.41	1856.20	6.06	0.0110
Error	16	4899.86	306.24	-	-
Total	18	8612.27	-	-	-

Appendix 3.14: Analysis of variance of the effect of height of the tiller of panicle (cm) on anther culture productions, established ACD lines in the soil from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	2	1901.01	950.50	4.15	0.0354
Error	16	3668.58	229.29	-	-
Total	18	5569.58	-	-	-

Appendix 3.15: Mean respons of companets of anther culture production in relation to tillerheight of the the tiller of donor panicle

Height (cm)	%Res A	% TGPl	%GPlSS	%EsPL
50-99	37.98ab	53.85b	53.50b	18.48ab
100-149	22.59b	27.02b	11.05b	8.18b
150-199	53.21a	96.79a	47.55a	24.37a

* means with the same letter are not significantly different.

Appendix 3.16: Analysis of variance of the effect of pollen stage on anther culture response per 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	6	4115.93	685.99	2.16	0.0959
Error	18	5712.83	317.38	-	-
Total	24	9828.77	-	-	-

Appendix 3.17: Analysis of variance of the effect of pollen stage on anther culture productions, calluses and sub-calluses developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	6	69709.14	11618.190	1.14	0.3791
Error	18	183297.30	10183.18	-	-
Total	24	253006.44	-	-	-

Appendix 3.18: Analysis of variance of the effect of pollen stage on anther culture productions, total ACD plantlets developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	6	56062.19	9343.70	2.11	0.1025
Error	18	79666.33	4425.91	-	-
Total	24	135728.52	-	-	-

Appendix 3.19: Analysis of variance of the effect of pollen stage on anther culture productions, albino plantlets developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	6	18471.50	3078.58	1.02	0.4422
Error	18	54190.75	3010.60	-	-
Total	24	72662.25	-	-	-

Appendix 3.20: Analysis of variance of the effect of pollen stage on anther culture productions, total green ACD plantlets developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	6	16758.20	2793.03	2.63	0.0523
Error	18	19148.79	1063.82	-	-
Total	24	35906.99	-	-	-

Appendix 3.21: Analysis of variance of the effect of pollen stage on anther culture productions, green plantlets transferred into strail soil from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	6	4493.17	748.86	2.31	0.0788
Error	18	5835	324.17	-	-
Total	24	10328.21	-	-	-

Appendix 3.22: Analysis of variance of the effect of pollen stage on anther culture productions, established ACD lines in the soil from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	6	2465.63	410.94	1.76	0.1634
Error	18	4191.70	232.87	-	-
Total	24	6657.34	-	-	-

Appendix 3.23: Analysis of variance of the effect of per-treatment on anther culture productions, green plantlets responded anthers from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	1	1017.24	1017.24	2.90	0.1036
Error	21	7378.80	351.37	-	-
Total	22	8396.05	-	-	-

Appendix 3.24: Analysis of variance of the effect of per-treatment on anther culture productions, number of calluses and sub-calluses developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	1	66.04	66.04	0.01	0.9360
Error	21	209734.51	9987.36	-	-
Total	22	209800.55	-	-	-

Appendix 3.25: Analysis of variance of the effect of per-treatment on anther culture productions, total ACD plantlets developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	1	146.95	146.95	0.03	0.8749
Error	21	121597.44	5790.35	-	-
Total	22	121744.39	-	-	-

Appendix 3.26: Analysis of variance of the effect of per-treatment on anther culture productions, albino plantlets developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	1	2720.40	2720.40	0.85	0.3670
Error	21	67191.41	3199.59	-	-
Total	22	69911.81	-	-	-

Appendix 3.27: Analysis of variance of the effect of per-treatment on anther culture productions, total green ACD plantlets developed from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	1	4131.90	4131.90	3.16	0.0898
Error	21	27435.26	1306.44	-	-
Total	22	31567.16	-	-	-

Appendix 3.28: Analysis of variance of the effect of per-treatment on anther culture productions, green plantlets transferred into sterile soil from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	1	533.76	533.76	1.28	0.2713
Error	21	8783.20	418.25	-	-
Total	22	9316.96	-	-	-

Appendix 3.29: Analysis of variance of the effect of per-treatment on anther culture productions, established ACD lines in the soil from 100 cultured anthers.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Treatment	1	240.04	240.04	0.87^{ns}	0.3604
Error	21	5765.24	274.54	-	-
Total	22	6005.28	-	-	-

II. Appendices of chapter 4

Appendix 4.1: Analysis of variance of plant height (cm) of ACD plants taken at the beginning of flowering, after 88 days grown in the field.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Replications	3	6699.9715	2233.3238	36.88	0.0001
ACD plants	87	35766.2536	411.1064	6.79	0.0001
Error	250	15139.5285	60.5581	-	-
Total	340	57425.8358	-	-	-

Appendix 4.2: Analysis of variance of tiller production capacity of ACD plants taken at the beginning of flowering, after 88 days grown in the field

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Replications	3	297331.5074	99110.5025	9.31	0.0001
ACD plants	87	7696853.5791	88469.5814	8.31	0.0001
Error	261	2777223.1915	10640.7019	-	-
Total	351	10771408.2781	-	-	-

Appendix 4.3: Analysis of variance of leaf shape ACD plants taken at the beginning of flowering, after 88 days grown in the field. (1 = rolled, 2 = nearly rolled, 3 = intermediate, 4 = partly expanded lamina and 5 = fully expanded lamina)

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Replications	3	3.8406	1.28020	9.28	0.0001
ACD plants	87	92.05392	1.05809	7.67	0.0001
Error	250	34.4927	0.13797	-	-
Total	340	130.5161	-	-	-

Appendix 4.4: Analysis of variance of leaf length

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Replications	3	3612.9328	1204.3109	126.04	0.0001
ACD plants	87	36321.6409	417.4901	43.69	0.0001
Error	1605	15335.3444	9.5547	-	-
Total	1695	55228.3774	-	-	-

Appendix 4.5: Analysis of variance of leaf width in ACD plants taken

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Replications	3	26.1263	8.7088	9.25	0.0001
ACD plants	87	4953.8206	56.9405	60.47	0.0001
Error	1604	1510.3103	0.9416	-	-
Total	1694	6489.1835	-	-	-

Appendix 4.6: Analysis of variance of flowering index of ACD plants in the field.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Replications	3	250.3796	83.4599	2.40	0.0743
ACD plants	38	9861.2865	259.5075	7.45	0.0001
Error	79	2750.5370	34.8169	-	-
Total	120	12843.4215	-	-	-

Appendix 4.7: Analysis of variance of the dry matter of herbage yield (g).

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Replications	3	1.63454	0.54485	26.46	0.0001
ACD plants	87	30.88531	0.35500	17.24	0.0001
Error	261	5.37519	0.02060	-	-
Total	351	37.89503	-	-	-

III. Appendices of chapter 6

Appendix 6.1. Analysis of variance of dry herbage mass g ($\log_{10}$ (x + 1)) of control genotypes at the first cut, before the imposition of drought.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Replication	1	143.3134	143.3134	1.20	0.3345
Environment	1	1293.5557	1293.5557	17.14#	0.0301
Rep (Env.)	1	75.4507	75.4507	-	-
Genotypes	2	2940.7652	1470.3826	12.33	0.0195
Geno × Env	2	437.5492	218.7746	1.84	0.2719
Error	4	476.8388	119.2097	-	-
Total	11	5367.4729	-	-	-

Variance ratio F calculated using replications within field as the denominator.

Appendix 6.2. Analysis of variance of log transfered harbage dry herbage mass of ACD genotypes at the second cut, (yield of 87 days), after 92 days of drought, with that at Cut 1 as covariate.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Replication	1	0.0009	0.0009	0.03	0.8610
Environment	1	2.4751	2.4751	17.30#	<0.01
Rep (Env.)	1	0.1430	0.1430	-	-
Genotypes	90	9.5317	0.1059	3.48	0.0001
Geno × Env	90	3.3782	0.0375	1.23	0.1180
Covriate	1	1.4277	1.4277	46.97	0.0001
Error	179	5.4407	0.0304	-	-
Total	363	68.637	-	-	-

Covriate = yield of cut 1 (prior to onset drought).

Variance ratio F calculated using replications within field as the denominator.

Appendix 6.3. Analysis of variance of log-transfered herbage dry herbage mass of ACD genotypes at the third cut, yield of 18 days, after 110 days of drought, with that at Cut 1 as covariate.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Replication	1	0.0015	0.0015	0.21	0.6479
Environment	1	0.1558	0.1558	45.02#	<0.0001
Rep (Env.)	1	0.0035	0.0035	-	-
Genotypes	90	4.1720	0.0464	6.37	0.0001
Geno × Env	90	0.9232	0.0103	1.41	0.0270
Covriate	1	0.0598	0.598	8.21	0.0047
Error	179	1.3024	0.00728	-	-
Total	363	13.4127	-	-	-

Covriate = yield of cut 1 (prior to onset drought)

Variance ratio F calculated using replications within field as the denominator.

Appendix 6.4. Analysis of log-transfered herbage dry herbage mass of ACD genotypes at the fourth cut, yield of 47 days, after 110 days of drought, with that at Cut 1 as covariate.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Replication	1	0.0008	0.0008	0.37	0.5449
Environment	1	0.0175	0.0175	33.60#	<0.01
Rep (Env.)	1	0.0005	0.0005	-	-
Genotypes	90	1.8672	0.0208	9.19	0.0001
Geno × Env	90	0.3937	0.0044	1.94	0.0001
Covriate	1	0.0037	0.0037	1.62	ns
Error	179	0.4043	0.0226	-	-
Total	363	4.5723	-	-	-

Covriate = yield of cut 1 (prior to onset drought), ns = not significant

Variance ratio F calculated using replications within field as the denominator.

Appendix 6.5. Analysis of variance of tiller production index (ratio of number of tiller at one day before end of drought (T2) per number of tiller at the beginning of drought (T1) with the use of number of tiller at the beginning of drought (T1) as covariant) at the end of drought, after 110 days of drought.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Replication	1	1233	1233	1.78	0.1836
Environment	1	56582	56582	60.55#	<0.001
Rep (Env.)	1	934	934	-	-
Genotypes	90	228844	2543	3.68	0.0001
Geno × Env	90	81785	909	1.31	0.0634
CV(T1)	1	15262	15262	22.06	0.0001
Error	177	122439	692	-	-
Total	361	749943	-	-	-

CV(T1) = Covariate of tillers number of cut 1 (prior to onset drought)

Variance ratio F calculated using replications within plot as the denominator.

Appendix 6.6. Analysis of variance of survival of ACD plants and control genotypes survival condition (scores from 0 to 15, see text) at the end of drought (after 77 days exposed to drought in a glasshouse).

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Blocks	1	0.2989	0.2989	0.036#	ns
Environment	1	404.8225	404.8225	48.82#	0.0001
Error (A) (Block×Env)	1	8.2927	8.2927	-	-
Genotypes	130	8927.4936	68.6730	5.89	0.0001
Geno × Env	129	5210.7518	40.3934	3.46	0.0001
Error (B)	1022	11918.743	11.662	-	-
Total	1284	30295.028	-	-	-

Variance ratio F calculated using blocks within experiment as the denominator.

Appendix 6.7. Analysis of variance of survival of ACD plants and control genotypes survival condition (scores from 0 to 18, see text) after 33 days recovery following 77 days drought experiment in an especial glass house.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio F	P_{H0} Pr>F
Blocks	1	6.5804	6.5804	0.283#	ns
Environment	1	4.6679	4.6679	0.201#	ns
Error (A) (Block×Env)	1	23.2592	23.2592	-	-
Genotypes	130	9758.5004	75.0654	3.71	0.0001
Geno × Env	129	5581.5638	43.2679	2.14	0.0001
Error (B)	1024	20698.545	20.213	-	-
Total	1286	42394.393	-	-	-

Variance ratio F calculated using blocks within experiment as the denominator.

Appendix 6.8. The correlation between herbage mass and tiller production capacity at the end of the drought under field conditions and herbage mass after recovery and survival score under glasshouse conditions at the end of drought period and after recovery of ACD genotypes and the three control genotypes. Data given are product moment correlation coefficients (r), the probability of the null hypothesis (H = 0) and the sample size.

Field drought experiment	Glasshouse drought experiment (survival score)	
	End of drought (SS = 0-15)	After recovery (SS = 0-18)
Herbage mass at the end of drought (Cut 3)	0.42442	0.36876
Probability Ho	0.0046	0.0150
Number of genotypes	43	43
Herbage mass after recovery following drought (Cut 4)	0.30528	0.26804
Probability Ho	0.0465	0.0822
Number of genotypes	43	43
Tiller production capacity at the end of drought (Cut 3)	0.41089	0.37141
Probability Ho	0.0062	0.0142
Number of genotypes	43	43

Appendix 6.9. The correlation between freezing tolerance (LT_{50}) and yield (mg/day) in the field, tiller production capacity (%) in the field; and survival score (Table 6.3) for ACD plants and the three control genotypes. Data given are product moment correlation coefficients (r), the probability of the null hypothesis (r = 0) and the sample size.

Variables	Cold tolerance (LT_{50})
PGR during 87 days of drought in the field (Cut 2)	-0.66566
Probability Ho	0.0001
Number of genotypes	48
PGR during the last 18 days of drought in the field (Cut 3)	-0.66778
Probability Ho	0.0001
Number of genotypes	48
PGR during 47 days recovery following drought in the field (Cut 4)	-0.75074
Probability Ho	0.0001
Number of genotypes	48
Tiller production capacity at the end of drought prod in the field (Cut 3)	-0.55942
Probability Ho	0.0001
Number of genotypes	48
Survival score after 77 days drought in the glasshouse (scores 1-15)	-0.35873
Probability Ho	0.0213
Number of genotypes	41
Recovery after 44 days following drought in the glasshouse (scores 1-18)	-0.34895
Probability Ho	0.0253
Number of genotypes	41

IV. Appendices of chapter 7

Appendix 7.1. Analysis of variance of % cell membraine damage flowing leaf desiccation of ACD plants and the three control genotypes.

Source of variation	Degrees of freedom	Corrected sums of squares	Mean square	Variance ratio of F	P_{H0} Pr>F
Test-days	1	6120.1	6120.1	88.76	0.0001
Genotypes	47	86292.6	1836.0	26.63	0.0001
G. × T.D.	46#	8490.6	184.6	2.68	0.0001
Error	93#	6412.3	69.0	-	-
Total	187	107315.619	-	-	-

ACD plant 51 was not tested on test day 2.

Appendix 7.2. The correlation coefficients between yield (mg/day), tiller production capacity (TPC) (%) at the end of the drought of drought experiment at the field (IGER 1994) and cell membraine damage (CMD) (%). Data given are the product moment correlation coefficients and the probability of the nul hypothesis (Ho) that correlation coefficients = 0. Cut 2 = first cut after 87 days drought, Cut 3 = yield during 18 days at the end of drought and Cut 4 = yield of 44 days during recovery following drought.

	Cut 2 (yield)	Cut 3 (yield)	Cut 4 (yield)	TPC
CMD (%)	0.029	0.030	0.061	-0.076
Pro Ho	0.846	0.841	0.680	0.608
n	48	48	48	48

After deleting those genotypes having zero yield.

	Cut 2 (yield)	Cut 3 (yield)	Cut 4 (yield)	TPC
CMD (%)	0.004	0.015	0.162	-0.076
Pro Ho	0.978	0.924	0.401	0.608
n	46	43	29	48

Appendix 7.3. The correlation coefficients between yield (mg/day), tiller production capacity (TPC) (%) at the end of the drought and total stomatal frequency on both leaf surfaces. Data given are the product moment correlation coefficients and the probability of the nul hypothesis (Ho) that correlation coefficients = 0. Cut 2 = first cut after 87 days drought, Cut 3 = yield during 18 days at the end of drought and Cut 4 = yield of 44 days during recovery following drought.

	Cut 2 (yield mg/day)	Cut 3 (yield mg/day)	Cut 4 (yield mg/day)	TPC (%)
Stomatal frequency	-0.38749	-0.62290	-0.10196	-0.30623
Pro Ho	0.3028	0.0731	0.7941	0.4229
n	9	9	9	9

V. Presentations

1. Ghamari Zare, A., M.W. Humphreys, H.A. Collin and W.J. Rogers 1994. Anther culture as a tool in utilising the interspecific hybrid, *Lolium multiflorum x Festuca arundinacea*. Proceeding of the VIII International Congress of Plant Tissue and Cell Culture, Firenze, Italy.

2. Ghamari Zare, A., M.W. Humphreys, H.A. Collin and W.J. Rogers 1995a. Use of anther culture for improved selection for cold tolerance in *Lolium multiflorum x Festuca arundinacea*. Journal of Experimental Botany, 46, 66.

3. Ghamari Zare, A., M.W. Humphreys, W.J. Rogers, M. Mortimer and H.A. Collin 1995b. Use of anther culture for improved selection for drought tolerance in *Lolium multiflorum x Festuca arundinacea*. Proceedings of the Inter Drought 95 - International Congress on Integrated Studies on Drought Tolerance of Higher Plants, Montpellier, France, VIII 44.

4. Humphreys, M.W., A. Ghamari Zare, I. Pasakinskiene and M-C. Kerlan 1996. The potential of anther culture derived plants from a *Lolium multiflorum x Festuca arundinacea* (2n = 5x = 35) hybrid as a novel forage grass crop. EUCARPIA, Fodder Crops and Amenity Grasses Section, 20th Meeting, October 7-10 1996 Radzików, Poland.

VIII INTERNATIONAL CONGRESS OF PLANT TISSUE AND CELL CULTURE
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ANTHER CULTURE AS A TOOL IN UTILISING THE INTERSPECIFIC HYBRID, *LOLIUM MULTIFLORUM* X *FESTUCA ARUNDINACEA*

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Italian ryegrass (*Lolium multiflorum*) and tall fescue (*Festuca arundinacea*) are two major agricultural species within the *Lolium-Festuca* complex. They present a range of complementary characters important in the development of consistently productive and persistent grass swards. Our aim was to obtain haploid and doubled-haploid lines from a fertile pentaploid (2n = 5x = 35) hybrid of autotetraploid *L. multiflorum* (2n = 4x = 28) x allohexaploid *F. arundinacea* (2n = 6x = 42) via anther culture, and to establish whether any of the lines combine the favourable characteristics of the two parental species. Anthers at the uninucleate stage were isolated from flowering shoots and incubated on a modified MS medium under lights at 25°C. Callus initiation took place, which was followed by transfer to regeneration medium for embryo and shoot formation. The shoots were grown to maturity and transferred to compost. More than 2,000 plantlets have been produced through androgenesis from which we have currently obtained approximately 500 established green plants. Chromosome observation of some of the regenerants showed that there was variation in the chromosome number. We are currently assessing the number and type of genetic combinations obtained, before evaluating their agronomic performance.

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Use of anther culture for improved selection for cold tolerance in *Lolium multiflorum* X *Festuca arundinacea* [CP3.13]

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Italian ryegrass (*Lolium multiflorum*) and tall fescue (*Festuca arundinacea*) are two major agricultural species within the *Lolium-Festuca* complex. They present a range of complementary characters important in the development of consistently productive and persistent grass swards.

Previously a pentaploid hybrid of autotetraploid *L. multiflorum* x allohexaploid *F. arundinacea* was to transfer drought resistance genes from fescue into the ryegrass species. In the present study, anther culture-derived plants of the same pentaploid hybrid were used as an effective method for selection of genotypes with enhanced cold or drought resistance above the levels available in ryegrass. Using chromosome doubling techniques, recessive genes which may govern some of the physiological processes involved, will be fixed in the homozygous condition.

A close relationship in the grasses between winter hardiness in the field and tiller performance in simulation conditions in a freezing tank containing ethylene glycol has been demonstrated at IGER. The anther culture-derived plants were placed in the freezing tank to determine their cold tolerance (LT_{50}).

The genotypes were exposed to temperatures ranging between -2°C to -14°C. All the tillers of one anther culture derived plant survived -14°C and two more were hardier than tall fescue plants(LT_{50} = -11.8°C) taken from the parental population. The *L. multiflorum* x *F. arundinacea* pentaploid hybrid had a LT_{50} = -10.6°C.

It is concluded that anther culture is an effective way of selecting traits governing stress tolerance in *Lolium-Festuca* hybrids and can be incorporated into conventional breeding programmes.

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USE OF ANTHER CULTURE FOR IMPROVED SELECTION FOR DROUGHT RESISTANCE IN *LOLIUM MULTIFLORUM* X *FESTUCA ARUNDINACEA*

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Italian ryegrass (*Lolium multiflorum*) and tall fescue (*Festuca arundinacea*) are two major agricultural species within the *Lolium-Festuca* complex. They present a range of complementary characters important in the development of consistently productive and persistent grass swards.

Previously a pentaploid hybrid of autotetraploid *L. multiflorum* x allohexaploid *F. arundinacea* was used to transfer drought resistance genes from fescue into the high quality ryegrass species. In the present study, anther culture-derived plants of the same pentaploid hybrid were used as a method to select for genotypes with enhanced drought resistance above the levels available in ryegrass. By means of anther culture and chromosome doubling techniques, recessive genes which may govern some of the physiological processes involved, can be fixed in the homozygous condition.

A total of 88 haploid plants derived from a single original hybrid were grown under rain shelters to assess their drought tolerance. Controls were provided by plants of *Lolium multiflorium*, *Festuca arundinacea* and the original hybrid *L. multiflorium* x *F. arundinacea*. A broad range of drought tolerance was found in the anther derived genotypes. However three haploid plants had a better yield than both parents by the end of the drought period. A number of parameters were measured on haploid plants showing extremes of susceptibility and tolerance to identify correlations between physiological adaptation and tolerance. Measurements were made on cell membrane stability under both desiccation and high temperatures. Ion release from leaves was used as a criteria for membrane damage. Measurements were also made on stomatal density and time of flowering. This assessment of drought tolerance will be repeated in June-July 1995

However our initial data does suggest that anther culture is an effective way of selecting traits governing stress tolerance in *Lolium-Festuca* hybrids to produce homozygous lines that can be incorporated into conventional breeding programmes.

THE POTENTIAL OF ANTHER CULTURE DERIVED PLANTS FROM A *L. MULTIFLORUM* X *F. ARUNDINACEA* (2N = 5X = 35) HYBRID AS A NOVEL FORAGE GRASS CROP

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ABSTRACT

Pollen microspores from a *Lolium multiflorum* (2n = 4x = 28) x *Festuca arundinacea* (2n = 6x = 42) hybrid were cultured and over 200 aneupolyhaploid green plants established. The initial hybrid contained one chromosome in all five homoeologous groups labelled by a distinct PGI/2 homoeoallele. Segregation of PGI/2 alleles in the male gametes and in the androgenic plants was an indicator of chromosome pairing preferences in the hybrid at meiosis.

The pentaploid *Festulolium* hybrid comprised of two homologous sets of *Lolium* (*Lm*) chromosomes and the polyhaploid set of *F. arundinacea* (one genome of *F.pratensis* (*Fp*) and two of *F. glaucescens* (*Fg* and Fg_1). There was a high degree of preferential chromosome pairing between the two homologous sets of *L.multiflorum* chromosomes and each androgenic plant contained a *Lolium* PGI/2 allele. Results supported earlier work indicating that gamete viability depended on the presence of a complete *Lolium* genome.

Genomic *in situ* hybridization (GISH) was carried out on mitotic chromosome preparations of two androgenic plants using as probe, total genomic DNA of *L.multiflorum*. Recombinants were observed between chromosomes of all three genomes in the pentaploid hybrid demonstrating the efficacy of the hybrid as a starting point in introgression breeding programmes.

Genotypes were identified with high levels of coacclimation to drought and freezing stress assessed under simulated conditions, in some cases in excess of the stress resistant *Festuca* parent used as control. Some *Festulolium* genotypes combined high stress tolerance and good agronomic performance and have considerable potential for development into novel cultivars.

Key words: Androgenesis; cold resistance; drought resistance; genomic *in situ* hybridization (GISH); *Lolium multiflorum x Festuca arundinacea* hybrid; meiosis; phosphoglucoisomerase (PGI/2).

INTRODUCTION

The *Lolium* and *Festuca* genomes in combination contain a range of complementary traits which together could be employed to produce a novel forage grass crop combining high nutritive value with persistency, abiotic, and biotic stress tolerance.

With anther culture, it should be possible to create homozygous lines from *Lolium* x *Festuca* F_1 hybrids which combine the high nutritive value of *Lolium* with the stress tolerance of *Festuca*.

A *Lolium multiflorum* (4x) x *Festuca arundinacea* (6x) pentaploid hybrid used previously in a backcrossing programme with *L.multiflorum* (2x) (HUMPHREYS, GHESQUIERE, 1994), was very responsive to cell culture (HUMPHREYS, DALTON, 1993), produced many inflorescences which were male fertile, and was considered as an ideal starting point for anther culture.

The *Festulolium* (5x) hybrid was constructed with one chromosome in all five homoeologous groups labelled by a distinct phosphoglucoisomerase (PGI/2) homoeoallele. The segregation of PGI/2 homoeoalleles in an androgenic population is considered a good indicator of chromosome behaviour at meiosis in the pentaploid *Festulolium* hybrid.

Two androgenic plants which display particularly high drought and freezing tolerance in combination with good agronomic characters, are cytologically characterized in detail, using genomic *in situ* hybridization (GISH).

MATERIALS AND METHODS

The pentaploid hybrid between *L. multiflorum* (*Lm*) (2n = 4x = 28) x *F. arundinacea* (*Fa*) (2n = 6x = 42) used for anther culture, was produced as described by HUMPHREYS (1989). In the pentaploid hybrid, two homologous chromosomes of *Lm* are labelled at the PGI/2 locus by two alleles "*a*" and "*b*", respectively. Three homoeologous chromosomes of *Fa*, are labelled by PGI/2 homoeoalleles "a^+", and "*c*", and "*e*", respectively. The "*c*" allele labelled a chromosome in the *F. pratensis* (*Fp*) genome, and the "a^+" and "*e*" PGI/2 alleles, chromosomes in each of the two *F. glaucescens* (*Fg* and Fg_1) genomes, of *F. arundinacea* (HUMPHREYS, 1995).

The PGI/2 phenotype of plants, all derived from anther culture from the *Lm* x *Fa* hybrid, was determined. The electrophoretic procedures used to separate variants at the PGI/2 locus in leaf samples of different androgenic plant genotypes were as described by HUMPHREYS (1995).

For anther culture all procedures were as described by WARD (1992). Great care was taken to isolate and record the origin of different embryoids as they were formed to eliminate any possible risk that two or more embryoids might derive from the same microspore and have the same genotype.

The poor root development of some androgenic plants, accompanied by the low frequency of mitotic cell divisions, limited the possibilities for detailed cytological investigation of the entire androgenic plant population. Approximate chromosome number of androgenic plants was determined using a flow cytometer and DNA values of plants of known ploidy number for comparison (MARIE, SPENCER, 1993).

DROUGHT TREATMENT

Clonal replicates of 88 randomly selected androgenic plants, together with their *Lm*, and *Fa* parents, and the *Lm* x *Fa* (5x) hybrid used for anther culture, were planted in the field under a rain-out shelter and irrigated daily to allow establishment. All genotypes were then cut and droughted continuously through the summer months over a 110 day period. During the drought treatment, plants were cut back twice. Dry matter production was

measured for each genotype following a final cut (cut 3) at the end of the drought treatment.

FREEZING TREATMENT

Clonal replicates of forty seven androgenic genotypes, all but one used in the drought experiment, together with *Lm*, *Fa*, and *Lm* x *Fa* (5x) parents were cold hardened in a controlled environment room at 2°C, 8hrs. day over 2wks. Single tillers of each genotype were then placed in plastic tubes in a freezing-tank at 2°C. Clonal replicates of each androgenic plant and of the control plants were removed from the tank as temperature was lowered at -2°C, -5°C, -8°C, -11°C, and -14°C. Individual tillers were then planted in shallow multitrays containing John Innes No. 3 compost in a controlled environment room at 15°C and 8hrs. day to score survival and to determine LT_{50} (lethal temperature for 50% of tillers from one genotype).

GENOMIC *IN SITU* HYBRIDIZATION (GISH)

Two androgenic plants, genotypes 193 and 219 (Fig. 1) with proven high drought and freezing resistance were selected for detailed cytological examination using genomic *in situ* hybridization. The techniques used followed procedures described by THOMAS *et al.*, (1994). Total genomic DNA of *Lm* and the two progenitors of *Fa* i.e. *Fp* and *Fg*, labelled with rhodamine-4-DUTP, was used as probe on mitotic chromosome preparations of the two anther culture derived plants. As blocking DNA, x40 probe concentration of *Fa* DNA was used with the *Lm* probe, and of *Lm* DNA with either of the *Festuca* probes. Following GISH, chromosome preparations were counterstained with DAPI.

RESULTS

Two hundred and twenty one green androgenic plants derived from different microspores and thus having different genotype, were successfully established in soil. The plants had very diverse morphology with contrasting leaf size, tiller number, growth habit, plant height, and root/shoot ratios. The plants also differed widely in their physiological characteristics, with plants of different growth rate, tillering capacity, and drought and freezing resistance.

(1) DROUGHT TREATMENT: (Fig. 1(a)

The drought resistant *Fa* parent produced more leaf and shoot growth than the *Lm* parent under severe drought conditions. The pentaploid hybrid, which is particularly vigorous and high tillering, produced more dry matter than the *Fa* parent. Only 7 androgenic plants produced more dry matter than the *Lm* parent. These 7 plants also produced more growth than the *Fa* parent but were less drought resistant than the pentaploid hybrid. Genotypes 219, 193 and 186 were the most drought resistant genotypes.

After 44 days recovery with daily irrigation subsequent to the drought period, the four most drought resistant androgenic plants maintained their superior growth rates.

(2) FREEZING TREATMENT: (Fig. 1(b)

The *Fa* parent (LT_{50} = -12°C) was considerably more cold resistant than the *Lm* parent (LT_{50} = -7°C). The *Lm* x *Fa* (5x) hybrid had a slightly inferior freezing resistance (LT_{50} = -11°C) to that of the *Fa* parent. The 47 androgenic plants displayed a

wide range of freezing resistance beyond the extremes of the *Lm* and *Fa* parents. Thirty six androgenic plants had inferior freezing resistance to the *Lm* parent and 3 plants (Genotypes 219, 193, and 186), superior freezing resistance to the *Fa* parent. No calculation for LT_{50} of Genotype 219 was possible since all tillers survived at -14°C.

(3) CYTOGENETIC STUDY:

The frequency of the 5 PGI/2 homoeoalleles between the 221 androgenic plants (Table 1) differed significantly ($X^2_{[4]} = 15.81$ $P<0.01$). There was no difference in frequency between any of the three *Festuca* alleles ($X^2_{[2]} = 1.61^{NS}$). The difference in frequency of the 5 PGI/2 alleles was explained by the high number of *Lm* "*a*" alleles, which were more frequent than the *Lm* "*b*" allele ($X^2_{[1]} = 6.36$ $P<0.05$).

Ninety eight percent of all plants carried at least one *Lm* PGI/2 allele with 10% carrying both *Lm* "*a*" and "*b*" alleles. The recovery of the *Fa* PGI/2 alleles was not as regular as *Lm*. Among the 221 androgenic plants, 30% carried no *Fg* PGI/2 alleles, and 14% carried both *Fg* alleles. The two *Fg* alleles were at equal frequency in the androgenic population ($X^2_{[1]} = 1.55^{NS}$). The *Fp* PGI/2 allele was recovered in 44% of the androgenic plants.

In Table 2, the frequency of the 5 PGI/2 alleles in the androgenic population was compared with their transmission via the pollen into the BC1 between *Lm* (2x) x (*Lm* x *Fa*) (5x) (HUMPHREYS,GHESQUIERE, 1994). There was no significant difference ($X^2_{[4]} = 8.43^{NS}$) in the frequency of PGI/2 homoeoalleles in the androgenic *Festulolium* and in the BC1 population. There was no selection in the BC1 against gametes with any *Lm*, *Fp*, *Fg* or Fg_1 PGI/2 alleles and chromosomes from the homoeologous set were transmitted in accordance with their frequency in the male gamete.

Approximate chromosome number of 128 randomly selected androgenic plants was determined using a flow cytometer. Chromosome number ranged between 14 chromosomes and >35 but the majority of plants were 14 to 21 chromosomes. There was little evidence amongst the androgenic population of spontaneous chromosome doubling, and the majority of plants were aneupolyhaploids.

(4) GENOMIC *IN SITU* HYBRIDIZATION:

Two androgenic plants, genotypes 193 and 219 (Fig. 1) which combined drought and freezing tolerance were chosen for a detailed cytological study using GISH. The *Lm* DNA as probe, hybridized preferentially onto *Lm* chromosomes, and to introgressed *Lm* chromosome segments on *Festuca* chromosomes. The *Lm* DNA probe partially hybridized to certain *Festuca* chromosomes but failed to hybridize with other *Festuca* chromosomes. The *Lm* genome is known to be structurally closer to *Fp* than to *Fg* (HUMPHREYS, 1995; PASAKINSKIENE *et al.*, 1996) and the *Festuca* chromosomes which partially hybridized with the *Lm* probe were likely those of *Fp*. The chromosomes which showed no evidence of hybridization with the *Lm* probe were *Fg*. Some chromosome segments with partial hybridization to the *Lm* probe were observed on otherwise unlabelled chromosomes. They were considered to be *Fg* chromosomes carrying *Fp* introgressions.

By using *Lm*, *Fp*, and *Fg* DNA as probes in turn, on different mitotic chromosome preparations of the two androgenic lines, the

chromosome composition of lines 193 and 219, could be determined. The conclusions derived from the different levels of hybridization observed between chromosomes of genotypes 193 and 219 and the *Lm* probe, were all confirmed. One of the *Lm* chromosomes in 219 is a large satellite chromosome identified as chromosome 2 with a long arm which is almost all *Fp*.

DISCUSSION

Development from microspores leads to monoploid plants and enables subsequent production of homozygous plants by chromosome doubling. This is an important step in many plant breeding programmes to improve the genetic gain during selection and achieve stability and uniformity during multiplication of selected material (e.g. MAYER *et al.*, 1995).

Microspores as products of meiosis represent a vast array of genetic variation. Through androgenesis, novel characters governed by gene combinations including recessive alleles, otherwise only rarely or never observed, may be revealed and selected. Very responsive genotypes to androgenesis such as the pentaploid *Festulolium* hybrid described, reveal much of the potential genotypic and phenotypic variation that can be generated through meiosis.

The *F. arundinacea* (*Fa*) parent of the *Festulolium* hybrid is more resistant to drought and freezing stress than *L. multiflorum* (*Lm*), the other parent. While most androgenic progeny of the *Festulolium* hybrid are less drought and freezing resistant than *Lm*, genotypes were identified with stress resistance in excess of *Fa*, the more stress resistant parent species. Three androgenic plants were found with coacclimation to both drought and freezing stress in excess of *Fa*.

The gene(s) controlling chromosome pairing in *Fa* are inactive in the hemizygous state (JAUHAR, 1975). The breakdown of the chromosome pairing control mechanism of *Fa* when hemizygous, leads to considerable chromosome pairing in F_1 *Lm* x *Fa* hybrids and the formation of interspecific chromosome associations (KLEIJER, 1984).

The cytogenetic analyses of the androgenic aneupolyhaploid plants described here provides clear insights as to the accuracy of predicted chromosome behaviour at meiosis. The presence of a *Lm* PGI/2 allele in 98% of androgenic plants provides strong evidence for preferential chromosome pairing and near regular disjunction between the two *Lm* genomes of the pentaploid hybrid. This adds to evidence from backcross breeding programmes (MORGAN *et al.*, 1988; HUMPHREYS, GHESQUIERE, 1994) that each gamete in the pentaploid hybrid contains a complete *Lm* genome. Ten percent of the androgenic plants carried homologous *Lm* chromosomes labelled "*a*" and "*b*" at the PGI/2 locus. The number of plants with both *Lm* alleles is an indication of the frequency of intergeneric chromosome pairing and of *Festulolium* multivalents. The near absence of plants with no *Lm* PGI/2 alleles is evidence that a complete *Lm* genome is required to provide gamete viability. Androgenic plants with either no *Fp* (56%) or no *Fg* (30%) PGI/2 labelled chromosomes were evidence that a complete *Festuca* genome is not essential for gamete viability.

No difference was observed between the segregation and recovery of the five PGI/2 alleles in androgenic plants with their transmission into the BC1 from *Lm* (2x) x *Festulolium* (5x) hybrids (see HUMPHREYS, GHESQUIERE, 1994). A lower than expected

recovery of a PGI/2 allele in the BC1 would be evidence of gametophytic selection through pollen competition or zygotic abortion which can occur in backcross breeding programmes and preclude recovery of certain gene combinations (HUMPHREYS, THOROGOOD, 1993).

The GISH chromosome study of two androgenic plants selected for coacclimation to extremes of drought and freezing stress proves that chromosomes from *Lm*, *Fp*, and *Fg/Fg*$_1$ genomes are present and can be distinguished. With *Lm* DNA as probe, chromosomes of *Lm* are readily distinguished. Chromosomes of *Fp* are identified since they share some common repetitive gene sequences with the *Lm* DNA probe which leads to low levels of hybridization with *Lm* DNA. The *Fg* chromosomes can be identified since they are not hybridized by the *Lm* DNA probe. The ability to distinguish all three genomes using *Lm* as probe facilitates a rapid cytological analysis of hybrids involving these species.

In addition to being able to discern chromosomes of the *Lolium* and *Festuca* species, reciprocal recombinants involving all three genomes were also observed. In genotype 219, a large *Fp* chromosome recombinant was observed which occupies nearly the entire length of the long arm of chromosome 2 of *Lm*. This chromosome arm of *Fp* is known to carry genes for drought resistance (HUMPHREYS, PASAKINSKIENE, 1996). It is considered likely that these *Fp* genes have contributed to the extreme drought resistance observed in genotype 219.

In order to utilise the variation obtained by anther culture, it will be necessary to restore fertility to selected androgenic plants and to incorporate them in breeding programmes. By chromosome doubling, preferential chromosome pairing between homologous chromosome partners will be encouraged. The selection for the pairing control gene(s) known to be located in one of the *Fa* genomes will enhance levels of preferential bivalent pairing.

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Table 1: The frequency of PGI/2 alleles in androgenic plants, each marking chromosomes from different homoeologous groups in the parent *L.multiflorum* x *F. arundinacea* (2n = 5x = 35) hybrid (PGI/2 = *a⁺abce*).

PGI/2 alleles					Total number	
Lolium		*Festuca*			Alleles	Plants
a	**b**	**a⁺**	**c**	**e**		
139 (26.6%)	100 (19.1%)	102 (19.5%)	97 (18.55%)	85 (16.25%)	523	221

Total X^2 = 15.81** ** = P< 0.01

Table 2: A comparison in the recovery of PGI/2 alleles in androgenic plants derived from a *L. multiflorum* x *F. arundinacea* (5x) (PGI/2 = *a⁺abce*) hybrid and their transmission into BC1* *L. multiflorum* (2x) x (*L.multiflorum* x *F. arundinacea*) 2n = 5x =35.

* The BC1 were derived from *L.multiflorum* (2x) ♀ x (*L.multiflorum* x *F. arundinacea*) (5x) ♂ (Humphreys and Ghesquière, 1994).

PGI/2 alleles		Androgenic Plants			BC1		
		Number	% alleles	Total No. plants	Number	% alleles	Total No. plants
a⁺	*Fg*	102	19.5%		27	12.4%	
a	*Lm*	139	26.6%		52	23.9%	
b	*Lm*	100	19.1%	221	53	24.3%	117
c	*Fp*	97	18.6%		50	22.9%	
e	*Fg*	85	16.2%		36	16.5%	
Total no. alleles		523			218		

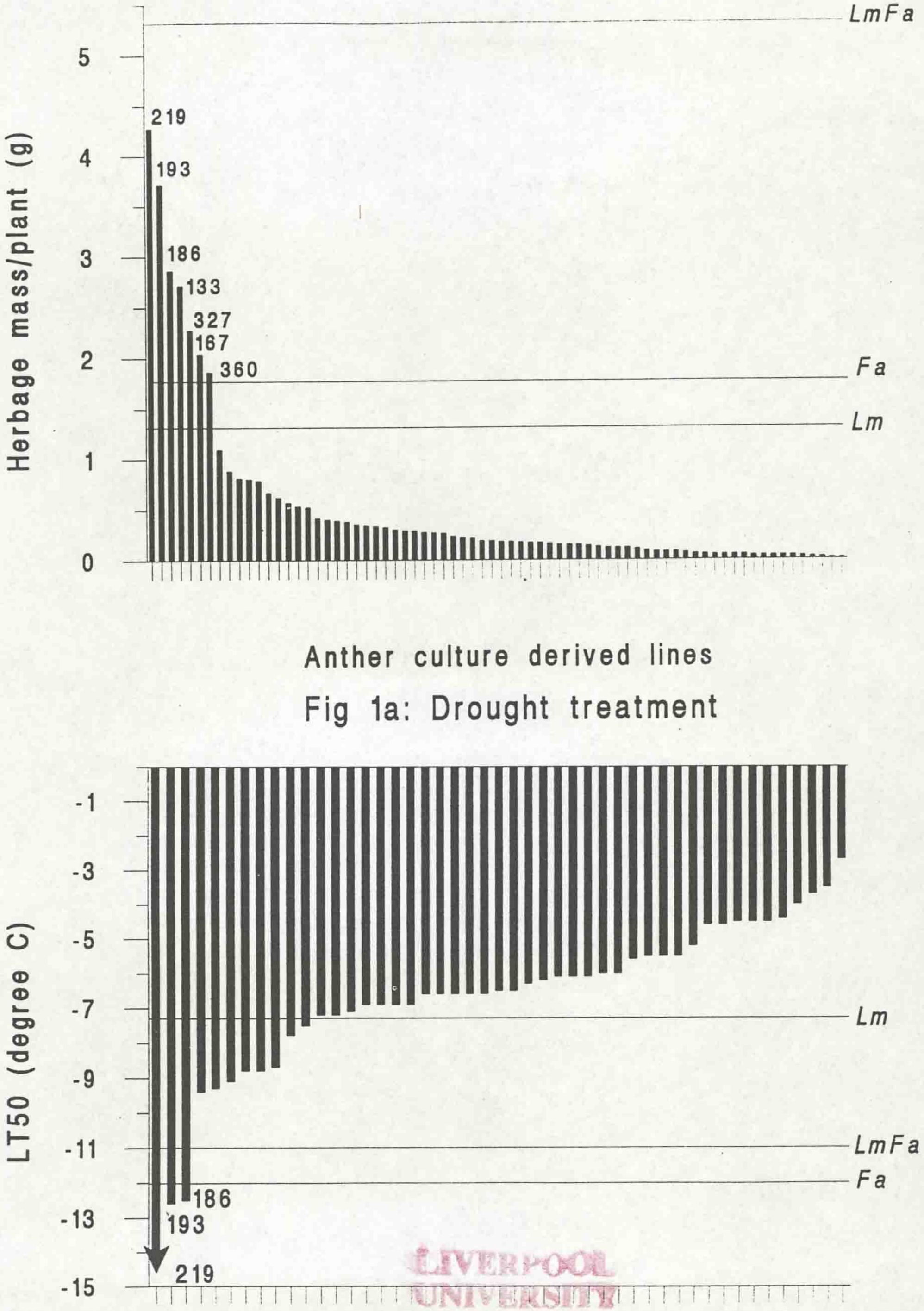
X^2 Comparison of PGI/2 allele frequency in androgenic and BC1 population. $X^2_{[4]} = 8.43^{NS}$

Fg = *F. glaucescens* PGI/2 allele
Lm = *L. multiflorum* PGI/2 allele
Fp = *F. pratensis* PGI/2 allele

Fig. 1(a). Leaf and shoot dry matter (gms.) of androgenic plants derived from a *L.multiflorum* x *F. arundinacea* (2n = 5x = 35) hybrid at cut 3 after 110 days continuous drought under a rain-out shelter.
Plants with superior drought resistance to *F. arundinacea* (the drought resistant parent) are indicated.

Fig. 1(b). $LT_{[50]}$ of androgenic plants derived from a *L.multiflorum* x *F. arundinacea* (2n = 5x = 35) hybrid assessed by insertion in a freezing-tank containing 50% (v/v) polyethylene glycol and water. Plants with superior freezing resistance to *F. arundinacea* (the freezing resistant parent) are indicated.

Lm = *L.multiflorum* parent
Fa = *F. arundinacea* parent
LmFa = *L.multiflorum* x *F. arundinacea* (5x) hybrid.



Anther culture derived lines

Fig 1a: Drought treatment

Anther culture derived lines

Fig. 1b: Freezing treatment