SOMACLONAL VARIATION IN *Linum usitatissimum* L.

(FLAX & LINSEED)

by

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A thesis submitted for the degree of

Master of Science

in the Faculty of Science at the University of Glasgow

December, 1993

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LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BA	N^6 - benzyladenine
BAP	Benzylamino purine
B5	Gamborg medium (1968)
FW	Fresh weight
KI	Kinetin
IAA	Indoleacetic acid
IBA	Indolebutyric acid
MS	Murashige and Skoog medium (1962)
NAA	α - Naphthylacetic acid
Ro	Regenerant generation from *in vitro* culture
R1	Produced from selfed Ro plants
R2	Produced from selfed R1 plants

ACKNOWLEDGEMENTS

Thanks are due to the Commission of the European Communities Directorate General for Science, Research and Development, for funding this project. I would like to acknowledge my supervisor, Dr G Marshall for his invaluable help during my studies. Without his support, advice and patience I would have been unable to complete this project.

I am also grateful to the technical staff, in particular Mrs Janice Hampson and Mr James Thomson for their help in carrying out the practical work. I also wish to thank Dr Jouan for his courtesy and in making me feel welcome during my visit to France with my supervisor and for exchanging material and information.

I wish to acknowledge the CPRO (Wageningen) for the twinning visit in 1988 where I learned the methodology applied to the androgenesis studies.

Lastly, I wish to thank Sheena Francis, secretary in the Department for typing my thesis and to Mr Tony Hunter and Mr Alistair Sword who helped and advised me on the statistical data.

SUMMARY

A study was undertaken to establish *in vitro* tissue culture systems for the production of callus, cell suspensions and regenerant plants from a selection of linseed and fibre flax cultivars. Reliable, prolific plant regeneration (25-60 % regeneration) was achieved from callus derived from the linseed cultivars Norlin and Linda; however, regeneration from fibre flax cultivars (Berber, Lidia, Natasja and Saskia) was lower (3-20%). In the production and regeneration of the cultivars *in vitro*, hypocotyl explants were grown on MS (Murashige & Skoog) medium supplemented with benzyladenine (BA) at 1 mg/l, napthaleneacetic acid (NAA) at 0.02 mg/l and 2.5 % sucrose. Regeneration was induced by transferring the calli to a MS medium supplemented with 1 mg/l of BA. Rooting was aided by the addition of 175 µg/l indole-3-acetic acid (IAA) to MS basal salts medium. The regenerant (Ro) plants thus produced displayed a range of somaclonal variation in terms of leaf shape, seed and flower colour, stem habit and flower sterility. Sufficient quantities of Ro's were produced from the linseed cultivar Norlin for replicated field trials of the R1's and R2's to be established and agronomic traits compared.

Suspension cultures initiated from callus cultures were difficult to establish especially of the linseed cultivars. The maximum regeneration obtained from a fibre flax cultivar (Saskia) was 6.0%, about one tenth of the level achieved with callus-based regeneration of Norlin (linseed). The callus-based system was also used to select regenerants (Ro's) following the *in vitro* stress of a sterile, crude culture filtrate derived from the pathogen *Fusarium oxysporum* f.sp. *lini.* Putative *Fusarium*-resistant somaclones were screened *in vivo* in the glasshouse for resistance to two isolates of *F.*

oxysporum f.sp. *lini*; however, only one of the 15 somaclones showed equivalent or improved resistance to the pathogen compared to the parent cultivar (Norlin).

In field trials conducted over two seasons, 47 R1 (plants derived from the Ro generation) and 20 R2 somaclonal lines (plants produced from the R1 generation) were compared to the parent cultivar Norlin for quantitative characters. Irrespective of the genotype, traits in the R1's and R2's were assessed on the basis of regression analysis as showing heritabilities of between 28 % and 64 %. Generally, the somaclonal variation assessed during these early generations revealed some detrimental traits, e.g. lower seed yield than the parent (control) cultivar and reduced 1000 seed weights, but a few of the lines were identified which had early or late flowering dates, improved seed yield and increased 1000 seed weights. It is concluded that somaclonal variation could be of value as an adjunct to the classical breeding of linseed.

CHAPTER 1

LITERATURE REVIEW

1. LITERATURE REVIEW

1.1 Description

1.1.1 Plant morphology

Within the genus *Linum,* the only species of agricultural importance is *Linum usitatissimum.* It is a crop grown in many countries for either fibre (flax) or oil (linseed).

Typically fibre flax produces single erect, wiry stems (40-50 cm) while linseed (30-40 cm) may tiller producing multiple stems from one plant. The leaves (3-4 cm) are disposed spirally along the length of the stem being lanceolate, glabrous with a marked mid-rib. Spontaneously, the plant produces ramifications at the upper part of the stem. This inflorescence is a racemose type. The number of flowers can vary depending upon the type of cultivar grown, flax or linseed. The linseed type possesses many more flowers than the flax type. Each flower has a diameter of 2-3 cm. The colour of the flower is genetically controlled which means that the flowers can range from white to dark blue depending of the set of genes involved (Comstock, 1965). The flowers of most cultivars are white or blue. The flower is composed of the set of 5 sepals (hairy or glabrous), 5 petals, 5 blue or yellow stamens (Comstock, 1965) and an ovary with 5 styles. The ovary has five chambers, with two ovules in each locullus which become separated by a false septum to produce ten chambers to give rise to ten seeds per fruit. The fruit is an indehiscent capsule for the majority of cultivars and is commonly known as a boll. The seeds are oval in shape, about 4-8 mm long. The colour of the seed coat is genetically determined, yellow, brown (light or dark), or black

depending on the cultivar. The seeds are of agronomic importance because they contain some 20% of proteins and 30- 40 % of oil in dry matter.

1.1.2 Plant anatomy

The green fine stem is the principal organ of the flax plant being characterized by a dicotyledonous infrastructure. There is a central pith surrounded by a ring of vascular bundles, xylem innermost, then cambium and phloem outermost. In turn, immediately outside each phloem bundle is a group of fibres which form a ring in the cortex. The fibre bundles run the whole length of the stem, each bundle consisting of overlapping strands measuring approximately 20 μm in diameter and 5 to 70 mm in length. About 10-40 strands make up the cross section. The middle lamella of the fibres are made of pectin and lignin deposits which vary with the age of the stem (Langer and Lane, 1982).

1.1.3 Origin and range of types

Flax and linseed are certainly old crops, grown extensively in many parts of the world such as Asia, Egypt, India, USSR, and Europe. In fact, the precise origin of the cultivated form is unknown although flax and linseed were used 1000 years ago by the Egyptians. Nowadays, both crops are grown in countries with temperate climates for fibre and oil production. The two principal uses are for fibre and oil production.

1.1.3.1 Fibre

The major trading countries of the EEC are Belgium and France. The extent of production in Europe for the 1992 cropping year is described in Table 1.1. It is apparent that the principle countries for fibre production are France and Romania. However, the importance of technological inputs and climatic factors are evident when a comparison is drawn between these two countries in term of the yields obtained. Clearly, France is the leading country in Europe for flax production and marketing.

While traditionally the fibres are used to produce high quality linen, they can be used in the series of non-textile products including non-woven fibres, thread, paper and compression moulding parts (Oosten, 1989, Marshall, 1993).

1.1.3.2 Oil (Linseed)

The world market in linseed has recently been greatly influence by a new production base in the UK. During the past 5 years the UK has increased its linseed area from less than 5,000 ha to over 130,000 ha (Table 1.2). This has had a dramatic effect on production in the two traditional producing countries, Canada and Argentina with reductions in the areas planted by a massive 60 %. UK farmers receive an area subsidy for growing linseed and since the climate is well-suited to linseed production, yields are high. In Europe the other main producing country is France (Table 1.2).

Table 1.1 The production of fibre flax in Europe*

Country	Area harvested (1000 ha)	Yield of fibre and tow (kg/ha)	Production (1000 tonnes)
Belgium	8	2880	22
Bulgaria	2	491	1
Czechoslovakia	12	504	6
France	37	1984	83
Italy	1	852	1
Netherlands	3	7765	20
Poland	6	340	2
Romania	86	485	42

* Source: FAO Yearbook 1992 (Volume 46)

Table 1.2 The production of linseed in Europe, Canada and Argentina*

Country	Area harvested (1000 ha)	Yield (kg/ha)	Production (1000 tonnes)
Belgium	10	900	9
Bulgaria	5	261	1
Czechoslovakia	12	697	8
France	37	537	20
Hungary	12	1038	14
Netherlands	2	2186	4
Poland	6	357	2
Romania	26	688	18
UK	139	1441	200
Argentina	216	866	187
Canada	253	1321	334

* Source: FAO Yearbook 1992 (Volume 46)

The main use of the seed is the oil, (linseed), used for industrial purposes such as paints, varnishes and linoleum manufacture (Oosten, 1989). Linseed oil is described as a yellowish-brown drying oil with a characteristic odour and bland taste. On exposure to air, the oil thickens and forms a hard varnish. A typical composition of linseed oil would be as follows: Saturated fatty acids - palmitic (5 %), stearic (4 %) and myristic (2 %); Unsaturated fatty acids - oleic (10-18 %), linoleic (24 %) and linolenic (36-50 %). Novel genotypes of linseed which are non-rapid drying, edible types are high in linoleic and oleic fatty acids (Green and Marshall, 1984). These novel oil-bearing linseeds will be commercialised in Canada during 1994 and may extend the opportunity to farmers to grow more oilseed crops.

1.2 Cell and tissue culture

The tissue culture of flax has not been extensively studied as a model crop. This is perhaps surprising since it is well known that the response of flax tissue *in vitro* is extremely rapid. The literature on flax tissue culture is rather restricted. Nevertheless, some authors have worked on this crop by using different techniques to obtain the flax lines of interest to the plant breeder. A group of Canadian researchers have worked on the improvement of linseed by using tissue culture techniques such as *in vitro* selection and subsequently genetic transformation via *Agrobacterium tumefaciens* (Jordan and McHughen, 1988). These authors have already selected a salt tolerant line (McHughen and Swartz, 1984) and a herbicide (chlorsulfuron) tolerant line (Jordan and McHughen, 1987). This work suggests that tissue culture techniques could be used as a new tool, as an adjunct to classical

hybridization and selection for the efficient and cost effective improvement of flax and linseed.

1.2.1 Tissue culture procedures

All the procedures of the tissue culture system are carried out under sterile conditions. Most researchers have sterilized seeds by rinsing in ethanol followed by surface sterilization with a solution of sodium hypochlorite. The last step consists of washes, several times with sterile distilled water. An alternative to ethanol as described by Singh and Govil (1982), used a mercuric chloride solution in distilled water (0.1%) and rinsed repeatedly with sterile distilled water.

1.2.2 Callus culture

Most researchers have used seeds as the starting material, which were sown in many different ways. Gamborg and Shyluk (1976) germinated the seeds on absorbent cotton with a 50% Hoagland's solution and incubated the seeds in the growth cabinet at 26 ^{o}C under cool white fluorescent light to produce satisfactory results. In these conditions, the seeds germinated in three or four days. Subsequent publications describe other methods which were easier to set up. For example, Singh and Govil (1982) sowed flax seeds on a simple agar medium while other researchers (Xiang-Can, Jones and Kerr (1989), use the medium described by Murashige and Skoog (1962). The requirement for light or dark during the germination is controversial. By reference to the literature on flax, the majority of workers omit light during this

particular phase. Germination temperatures typically range between 20 to 26 ^{o}C.

Few studies have been done on *in vitro* morphogenesis of different sources of explants. The work of Gamborg and Shyluk (1976) brought some information about the morphogenesis of flax *in vitro*. These workers determined that hypocotyls could be used to induced callus induction and growth. However, this did not necessarily result in the regeneration of complete plantlets.

Once the seeds are germinated, cotyledons, hypocotyl segments, meristem, and portions of the stem are typically used as explants to induce callus formation. The hypocotyls of flax have nearly been exclusively used to produce undifferentiated cells e.g. callus. Hypocotyl segments were of variable size, from 0.4 to 2 cm in length. Generally, the explant is positioned on the medium horizontally by applying a slight pressure (Gamborg and Shyluk, 1976).

It has also been possible to induce callus formation from different parts of the flax plant. Rybczynski (1975) showed the possibility of obtaining callus from cotyledons by adding a combination of auxin and cytokinin (Kinetin: NAA at the ratio 10:1). Murray, Handyside and Keller (1977) concluded that segments of stem could be used to regenerate plantlets. They took stems from donor plants at the pre, early, and mid-flowering stages. Their studies have proved that organogenesis was feasible. In addition, these authors pointed out that the shoots formed on same explant originated from four different sites: site 1 corresponded to the epidermal layer on the upper part

of the explant, site 2 was the epidermal layer that persisted over outgrowth, site 3 was the lower surface of the explant and site 4 was defined as the callus tissue at both ends of the explant.

Murray *et al.*, (1977) also noticed that the age of the donor plant had a significant effect on shoots regeneration since no regeneration occurred if plants were beyond the flowering stage. Chlyah, Squalli-Khallil and Chlyah (1980) observed a variability in the morphogenic expression in the stem segment depending on the age and the physiological state of the plant taken as an explant. Normal shoots were obtained from young plants and modified shoots when taken from the upper part of an older plant. In their studies excised hypocotyls from 15 days old plantlets and segments of stems of 30, 60 and 75 days old were grown in Hoagland's solution. Explants were taken at three levels on young and old stems. They found that hypocotyls and young stems were able to produce shoots with subsequent elongation like normal seedlings from epidermal origin in large numbers, whereas on older plants no normal shoots were found. A different type of bud appeared on the median or apical level of older plants. These buds did not elongate and produced some white leaves. Lane (1979) used axenic meristem tips and hypocotyl segments to study their response at different levels of cytokinins alone or in combination. He determined that BA alone used at 0.1 µM encouraged shoot proliferation whereas NAA alone inhibited shoot proliferation as low as 0.05 µM. At higher levels of NAA (10 µM) it initiated root formation. The response was similar for both meristems and hypocotyls. Finally, Australian researchers (Xiang-Can *et al.*, 1989) successfully regenerated shoots from root induced callus.

1.2.3 Regeneration systems

The regeneration of plantlets from undifferentiated tissues can generally be induced by adding cytokinin into the medium or in combination with an auxin. Rybczynski (1975) investigated the effect of several concentration ratios of kinetin and NAA on regeneration from cotyledon explants. At a ratio 10:1 cotyledon cells produced roots, callus tissue and shoots. By decreasing the concentration of kinetin ten-fold, only root formation occured. Gamborg and Shyluk (1976) found direct shoot formation on hypocotyls when zeatin was used in combination with auxin. Callus regeneration occurred with BAP (2 mg/l). Lane (1979) found that shoot initiation was induced on hypocotyl segments by using 0.1 BA (μM). Jordan and McHughen (1987) induced shoot formation with a concentration of BA (1 mg/l) from callus tissue. Lastly, Xiang-Can *et al.* (1989) induced shoot formation from roots by modifying the medium with NAA (0.02 mg/l), adenine (20 mg/l) and 500 mg/l of cefotaxime.

1.2.4 Suspension culture

Cell suspensions can be obtained by transferring pieces of callus into liquid callus induction medium. This system has the advantage over callus culture that separated cells may be subsequently subjected to a stress or selection pressure from a herbicide or disease producing toxins. It appears that little work has been done on flax because of the difficulty to regenerate shoots from cell suspensions. Gamborg and Shyluk (1976) obtained cell suspensions of the flax cultivar Redwood but no regeneration occurred on medium free of growth hormones.

1.2.5 Protoplast culture

The genus *Linum* is rich in variability. It is composed of approximately 40 species which differ in chromosome number (Gill, 1987). An alternative approach could be the production of somatic hybrids by using protoplasts. A few reports indicate that this method could solve the problem of interspecific crossings. Firstly, Gamborg and Shyluk (1976) obtained protoplasts from flax hypocotyls but no subsequent morphogenesis *in vitro* was noticed. Barakat and Cocking (1983) produced protoplasts from different parts of the seedling of one cultivar. Shoot formation from cotyledon-derived protoplasts was also possible. The same authors have successfully regenerated two *Linum* species. Shoot regeneration was obtained on MS media containing 0.05 mg/l NAA and 0.5 mg/l BAP. After several subcultures on this medium a high frequency of shoots was obtained.

1.3 Application of *in vitro* systems for somaclonal variation

Variation generated by tissue culture has been termed somaclonal variation (Larkin and Scowcroft, 1981). These authors defined a tissue culture cycle as a process that involves the establishment of dedifferentiated cells known as callus culture and subsequently the regeneration of a whole plant. Regeneration may occur following one or several subcultures in a particular envinroment (tissue culture vessel) associated to a certain regime of nutrition (growth hormones, explant source, balance of macro and micro nutrient, vitamins).

1.3.1 Callus culture

Callus culture is a mass of cells obtained from various parts of the plant (root, cotyledon, stem, leaf, microspore, embryo). Placed in a suitable environment the explant will produce disorganized growth which can be maintained for an extended period of time and then regeneration of a plant can be achieved via one or several cycles *in vitro*. As reviewed by Skirvin (1978), tissue culture can induce variation. Changes such as growth habit, colour, variability in organogenesis, plantlet morphology were observed for a number of crops during tissue culture. Orton (1980) compared callus types of an interspecific hybrid of *Hordeum* with respect to chromosomal constitution and regeneration capacity. It was found that hard slow-growing calli were stable with respect to chromosome number and retained their capacity to regenerate shoots. By contrast, friable callus growth from uncentralized fast-growing calli exhibited a high proportion of chromosomally aberrant cells and could not be regenerated. As Bayliss (1980) specified, chromosomal instability seems to be the rule in tissue culture for a wide range of crops. He showed that chromosomal abnormalities were frequent in tissue culture for a large number of genera whatever the system used (callus culture, suspension culture). Polyploidy was frequently reported. Ahloowalia (1983) found that plants regenerated from a single callus of an interspecific hybrid between diploid *Lolium multiflorum* and tetraploid *Lolium perenne* and were all triploids (21 chromosomes) after the first cycle *in vitro* but noticed abnormalities in plants which were regenerated from old callus (10 subcultures=600 days) such as polyploids (28 chromosomes) and aneuploids (29 chromosomes).

Skirvin (1978) compared plants of five *Pelargonium* cultivars obtained from cuttings derived from different parts of the plants (stem, petiole, roots) with plants regenerated from callus cultures. Stem-cutting derived plants were all uniform. Those from roots were more variable depending on the cultivar, whereas plantlets regenerated from calli were very variable compared to their parent for many characters such as plant size, leaf and flower morphology, anthocyanin pigmentation and essential oil composition. After a recurrent selection *in vitro* he found that the variation previously observed decreased and that stability was observed for the new traits for some lines. Skirvin investigated further the origin of these mutations and concluded that they were due to segregation of chimerical tissue, euploid changes which may involve simple gene mutations.

In tomato, Evans and Sharp (1983) regenerated 230 plants from leaf explants of a processing tomato cultivar UC82B. In R1, they observed mutations for fruit colour (tangerine), male sterility, green base, jointless character, growth habit indeterminate, mottled foliage. In total 13 mutations were found in the progeny of certain lines. The R1 segregations were compared with theoretical ratio and it was stated that they were heritable in a simple manner. These mutations occurred before shoot formation as regenerants were derived from a different leaf. Tangerine virescent mutant was crossed with a known tangerine genotype. The F1's were all tangerine and this suggested that the allelic mutation occurred during the tissue culture phase. In addition, to single nuclear changes, cytoplasmic mutations have been reported. In maize, in vitro selection for resistance to southern corn blight (*Drechslera maydis* race T) was carried out on a cytoplasmic male sterile line of maize using culture filtrate of the selecting agent. Plants were regenerated from

selected callus. All the plants were fertile and resistant to the disease which demonstrated that such cytoplasmic changes can occur. Recent reports suggested that biochemical modifications were obtained from a cycle *in vitro.* Allicchio, Antonioli, Graziani, Roncarati and Vannini (1987) regenerated plants from the potato cultivar Spunta from leaf callus. They were analyzed for 3 isoenzyme systems, glutamate oxaloacetate transaminase (GOT), alcohol dehydrogenase (ADH) and esterase (EST). In the regenerated plants and in the tubers of the first vegetative propagation they noticed that many of the plants did not possess ADH or GOT which were present in the parent. They determined that these changes were stable for ADH but not for GOT. All regenerated plants possessed EST not present in the parent. They suggested that these changes were due to DNA rearrangements in culture.

1.3.2 Suspension culture

Suspension cultures may have the advantage over callus to produce single cell colonies which allow easier indentification of the mutants. Groose and Bingham (1984) regenerated alfalfa plants (*Medicago sativa*) from callus culture and suspension culture of 2 tetraploid cultivars which were heterozygous for four traits: anthocyanin pigmentation in the flower, multifoliate leaf trait, ability to regenerate from tissue culture and cytoplasmic sterility conditioned by nuclear fertility restorer genes. After one cycle *in vitro* 21% of the regenerants were variant for one or several traits. They found that some 11% of the plants lost one or more chromosomes during this phase ranging from 28 to 31 but that 60% of the regenerated plants exhibited kariological changes. An unstable white flowered recessive mutation was

recovered. The freqency of somaclonal variants was about equal for plants regenerated from cell suspension and callus culture.

1.3.3 Protoplast culture

Protoplasts are defined as cells without their membrane, which has been eliminated by enzyme digestion. These structures can develop into a whole plant, in a few cases directly, or via a callus phase. Regenerated potato (*Solanum tuberosum*) plants have been found more variable than the parent plant (Sree Ramulu, Dijhuis and Roest, 1983). These authors regenerated a large number of plants (620) and noticed that 64% of these were normal looking whereas 32 % differed in a number of morphological traits like short plant, small leaves, shrivelled leaves. Their results suggested that these changes were mainly the consequence of polyploidy. They proposed that because sister plants originated from the same callus were different in phenotype and/or ploidy level the mutations arose during the tissue culture phase. Johnson, Stateville, Schlarbaum and Skinner (1984) regenerated alfalfa plants from two tetraploid cultivars named RS-K1 and RS-K2 and compared them to their respective parent. These authors have observed a shift in chromosome number for the two populations at a frequency of 30 and 45 % for the two populations respectively and also aneuploids and translocations. The somaclones were observed in field conditions for agronomical characters such as winter damage, forage yield. A wide range of variation was present but was considered of no practical value.

1.3.4 Anther and microspore culture

This method is designed to enable the plant breeder to produce homozygous lines in a very short period of time. Androgenesis has been successfully used in some crops such as *Capsicum annuum* (Dumas de Vaulx, Chambonnet and Pochard, 1981), *Solanum melongena* (Dumas de Vaulx and Chambonnet, 1982), *Brassica oleracea* var. *acephala* (Keller and Amstrong, 1981). Observations of haploid populations has permitted the detection of variation induced by tissue culture: morphological characters like yield reduction, alkaloid content were found in *Nicotiana tabacum* (Brown and Wernsman, 1982). Matzinger and Burk (1984) detected cytoplasmic mutant for altered temperature sensitivity in *Nicotiana tabacuum.* These findings confirmed that as termed by Evans and Sharp (1986) gametoclonal variation occurrs in anther culture and that these changes have a genetic explanation.

Nichterlein, Umbach and Friedt (1991) have successfully regenerated haploid and diploid plants from the linseed cultivars Hella and Atalante. Shoot and root morphogenesis was induced in callus using a modified N6 medium containing zeatin (1 mg/ml) then rooting on modified B5 or MS media containing NAA (0.1 mg/ml). Environmental conditions for the donor plants optimised shoot regeneration when a 16 h photoperiod and 14 ^{o}C day/8 ^{o}C night temperature was used.

As an alternative method, microspores have been isolated from fibre flax (Metz-Monteiro, 1991, Pers. comm) and linseed cultivars (Nichterlein *et al.*, 1991). This technique requires a significant amount of additional research to optimise the combination of genotype/environment necessary to produce

haploid regenerants. Nichterlein *et al.* (1991) obtained embryoids which then developed calli and were induced to produce roots and shoots. The ploidy level was not established; however, morphological characters suggested that regenerants were doubled haploids.

1.4 Breeding requirements for flax and linseed

Due to the revival of interest in non-food or industrial crops of late there are in fact several useful reviews of the current breeding requirements for flax and linseed, regardless of the ways in which they may be delivered i.e by conventional methods, somaclonal variation, biotechniques or even genetic engineering. Reviews have been published by Fouilloux (1989), Friedt, Nichterlein and Nickel (1989), Marshall (1992, 1993) and McHughen (1992).

In summary, the major characteristics in fibre flax which are important include improved fibre yield and quality (fine fibres), resistance to lodging and resistance to diseases (especially *Fusarium oxysporum* f.sp. *lini*). In linseed improved oil yield and the introduction of novel linseed oils, including a new edible oil (Green and Marshall, 1984, Green, 1986, Nichterlein *et al.*, 1988, Rowland, 1991) are currently receiving priority. Resistance to linseed diseases e.g. *Alternaria linicola* and *Oidium lini* are also receiving attention.

1.5 Objectives of this research

This project was designed to undertake the following:

1. Devise *in vitro* systems for flax and linseed cultivars which would allow them to be regenerated from callus and suspension systems.
2. Assess the regenerant plants or somaclonal lines in a series of field studies to determine the value of the somaclonal variation which was displayed.
3. Develop and assess an *in vitro* system to select and screen somaclonal lines of linseed which had been subject to fungal extracts from *Fusarium oxysporum* f.sp. *lini.*
4. To estimate, where seed supplies permit the heritabilities of some agronomic traits in somaclonal lines of linseed e.g. plant height and seed yield.

CHAPTER 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Callus culture

2.1.1 Sterilisation

Seeds were surface sterilised in ethanol (70%) for one minute, followed by a wash in sodium hypochlorite (1%) for 20 minutes, and then washed in sterile distilled water. Seeds were germinated on tap water agar under sterile conditions in the dark at 21^{o}C. After five days hypocotyl segments (0.4-0.8 mm) were isolated and placed on Murashige and Skoog medium (1962), supplemented with 1 mg/l of BA and 0.02 mg/l of NAA and 2.5 % sucrose in petri dishes which were sealed with clingfilm and finally placed in an incubator with 16 hours light period at 21^{o}C (light intensity equivalent to 81.6 µmol. $m^{-2}.s^{-1}$).

2.1.2 Growth

The cultivars selected were (1) fibre types: Berber, Lidia, Natasja and Saskia and (2) linseed types: Linda and Norlin with callus growth maintained in 9.0 cm diameter petri dishes. For all the cultivars an attempt was made to maintain callus growth on MS media by transferring to fresh media every 21 days.

It was apparent that only Norlin, Natasja, Saskia and Linda required to be transferred to a regeneration medium (MS supplemented with 1 mg /l of BA, 2.5% sucrose and 0.8 % of agar, pH 5.80) in order to stimulate shoot

initiation. Thus Berber and Lidia were maintained after 21 days on fresh callus medium to continue development.

2.1.3 Regeneration

The cultivars selected were Norlin, Natasja, Saskia and Linda to establish a method to regenerate flax varieties which did not respond in the first growth phase (2.1.2). Callus was obtained by placing the hypocotyl explants on Murashige and Skoog medium (1962) supplemented with 1 mg/l of BA, 0.02 mg/l of NAA, 2.50% of sucrose, pH 5.80. After 15 days of culture the explant produced sufficient quantities of callus to allow transfer to a regeneration medium containing basal salts: MS supplemented with 1 mg/l of BA. Among linseed cultivars only Norlin needed to be transferred to a regeneration medium to initiate shoots and the same medium was used to regenerate the cultivar Linda. For flax cultivars (Natasja, Saskia) three media were used: (i) Basal salts Murashige and Skoog (1962) supplemented with 1 mg/l of BA (previous regeneration media), (ii) MS+1 mg/l of KI and (iii) MS+1 mg/l of KI, 2 mg/l of IBA. Calli were also maintained on fresh callus induction medium for all the cultivars. The temperature was maintained at 21 ^{o}C with a photoperiod of 18 hours (light intensity, 99.2 µmol m^{-2} s^{-1}), 8 hours dark until regeneration occurred (3 weeks).

2.1.4 Rooting

Regenerant plantlets of the cultivars Norlin, Linda, Natasja and Saskia were transferred into sealed culture jars capped with polyethylene lids and grown on MS media without growth hormone supplement in a controlled

environment cabinet at 25 ^{o}C with a photoperiod of 16 hours. The regenerant plants in jars were assessed for phenotype variation in terms of shoot pigmentation (red, pigmented, translucent).

Rooting was obtained by first transferring to a medium free of growth hormone (Murashige and Skoog basal salts) for a period of one week to establish sufficient shoot elongation. Subsequently, shoots were transferred into Murashige and Skoog (basal salts) containing an auxin (IAA 175 µg/l) (Gamborg and Shyluk, 1976). The temperature was decreased to 21 ^{o}C to encourage root formation (controlled environment as previously described).

Surviving plants from all the cultivars were removed from the culture jars, the roots washed in distilled water and transplanted into a non-sterile compost consisting of one part of volcanic ash (perlite) to one part of coarse peat. Individual plants were transferred to a growth room at 20 ^{o}C, 16 hour photoperiod and the shoots were covered with top vented clear acrylic domes. This was essential to prevent desiccation of the young developing plants. The pots were watered as required and given a liquid nutrient solution feed (Solinure ex Fisons) at seven day intervals. Finally, the surviving plants were transferred to a controlled environment cabinet (Conviron) maintained at 20 ^{o}C, 18 hours photoperiod (light intensity, 152 µmol m^{-2} s^{-1}) and 80% relative humidity. Following the initiation of flowers, the plants were allowed to mature until the seeds bolls were ripe. At this time the bolls for each plant were removed and stored in dry conditions until required for seed extraction and sowing.

2.1.5 Assessments of growth in the Ro generation

The leaf, shoot, flower and seed morphological characteristics of the Ro plants (Norlin, Linda, Berber and Lidia) were compared to those of the parent cultivar grown under similar controlled environment conditions from seedling emergence through to maturity. Special note was made where characteristics were transient in nature.

2.2 Suspension culture

2.2.1 Initiation of suspensions

Ten pieces (2 g FW) of 21-31 days old callus of Lidia, Linda, Natasja, Norlin and Saskia were inoculated into 50 ml of liquid MS medium (as per callus growth) and placed in 250 ml conical flasks. There were five replicates for each cultivar. The flaskswere placed in an orbital incubator (140 cycles per minutes) at 25 ^{o}C with a 18 hours photoperiod supplied by fluorescent tubes. After one week the pieces of calli were removed and added to 50 ml of fresh medium. New suspension cultures were initiated every three weeks in this manner. Each culture was supplemented with fresh medium every week by removing 10-20 ml of the supernatant and adding 10-20 ml of fresh medium. Records were maintained according to the general appearance of each culture grown over a 9 weeks period.

2.2.2 Growth and regeneration

Various media were used to determine the ability of cells to regenerate. The second suspension culture medium was supplemented with 1 mg/l of BA

alone and the third medium was supplemented with 1 mg/l of BA and 0.5 mg/l of NAA and placed into 250 ml conical flasks. There were three replicates. The flasks were placed in an orbital incubator (140 cycles/min) at 25 ^{o}C with a 18 hours photoperiod supplied by fluorescent tubes. After one week the pieces of calli were removed by filtration through a fine metal mesh and 10 ml of the solution were removed and added to 50 ml of fresh medium. The same medium was used for the subcultures. Then after 7, 14 and 21 days of culture on any of the media used microcalli were plated on to different regeneration media as per 2.1.3. Suspensions were also removed and plated on solid agar.

2.3 *In vitro* selection

2.3.1 Fungal identification

Fusarium oxysporum f.sp. *lini* was isolated from stems harvested on an experimental field in Belgium. Stems of the suceptible cultivar Regina were collected in the field from 10 plants. Stems were surface sterilized with 70% alcohol for one minute, and then placed in a solution of sodium hypochlorite for 5 minutes and finally rinsed three times in sterile distilled water (10 minutes each). Stem sections of 6 cm in length were cut longitudinally and placed on selective agar containing antibiotics in 90 mm petri dishes. Stem sections were incubated at 25 ^{o}C with a 12 hour photoperiod.

The fungus was identified using 4 characteristics: growth rate, macroconidia, microconidia and the colour of the fungus. These characteristics were compared with the literature. This work was conducted at CPRO (the Netherlands) by Ir.Th.P Straathof. Further studies were done in order to

authenticate the specificity of the strains by CAB International Mycological Institute, Kew, England. It was also decided to test these strains *in vivo*.

2.3.2 Fungal culture

Agar plate cultures of *Fusarium oxysporum* were prepared with the following composition (g/l), peptone (2.0), glucose (2.8), magnesium sulfate (1.2), potassium dihydrogene orthophosphate (2.7), agar (20.0). Following incubation for 14 days at 20 ^{o}C in the dark, the discs of inoculum were placed in each large flasks in the light (2.1) containing the following growth media (g/l), glucose (30.0), sodium nitrite (2.0), potassium dihydrogen orthophosphate (1), potassium chloride (0.5). The inoculum was incubated in a rotary shaker at 23 ^{o}C for 5 days and the concentrations of conidia adjusted to 10^{-6} ml^{-1}.

2.3.3 Fusarium *in vitro* selection

Callus cultures were obtained as per 2.1. After 15 days of callus growth, pieces 3 mm in diameter were placed on Murashige and Skoog basal salts medium (Sigma Ltd) supplemented with 1 mg/l of BA and 0.02 mg/l of NAA. The medium was then sterilized at 120 ^{o}C for 15 minutes. The culture filtrate solution of the strain A10 was prepared as per 2.3.2 and filter sterilized with single use filter of 2µm (Minisart NML 16534) and added to the callus induction medium when it was warm (40 ^{o}C). The following concentrations were used 0, 2, 4, 5 % v/v (75 calli per concentration) and made up with Murashige and Skoog basal salt mixture as per 2.1.2. For the first selection

cycle, culture were incubated at 21 ^{o}C (16 hours photoperiod) for a duration of 10 days, and they were subsequently subcultured on medium supplemented with higher concentrations 2, 5 and 10 % v/v of fresh *Fusarium* culture filtrate. Cultures were maintained for another 10 days before being transferred onto regeneration medium as per 2.1.3 until regeneration occurred within 1-3 weeks. The putative regenerants were raised as described earlier (2.1.2).

2.3.4 *In vivo* bioassay

Callus was initiated from hypocotyl tissue derived from the cultivar Norlin and grown on MS media which incorporated sterile crude culture filtrate produced by the strain A10 of *Fusarium oxysporum* f.sp. *lini.* Calli were treated with a range of culture filtrate concentrations and over different periods of *in vitro* selection of 1-3 cycles of 20 days. Finally, surviving calli were regenerated and the Ro putative *F.oxysporum* f.sp. *lini* resistant lines screened as follows.

Fungal cultures were obtained as described above (2.3.2). Vermiculite (medium grade) was washed thoroughly in distilled water and placed in 12 cm diameter pots. Seeds of the somaclonal lines plus control varieties (Norlin=moderately resistant, Norlin Ro=unknown, Regina = suceptible cultivar) were sown (10 per pot) to a depth of 1 cm and 15 ml of inoculum added per pots. Nutrient solution was provided from the base of each pot to the germinating plants which were grown in glasshouse (15-20 ^{o}C). Each line consisted of two replicates with one non-inoculated control. Germination was recorded in each pot together with the development of

disease at 20 and 40 days after planting. The experiment was randomised in a block design and repeated once. The data recorded, i.e. percentage of emerged plants which remained healthy, were converted by angular transformations and subject to analysis of variance (Genstat 5.2).

2.4 Field trials

2.4.1 Field trial (1989)

In these studies, the symbol Ro (after Edallo, Zucchinali, Perenzin and Salamini, 1981) is used to denote the regenerant generation. Selfed seeds of the Ro generation produced plants for the R1. The bulk seeds produced from the R1 line was sown in the following year to produce an R2 line.

R1 plants were raised in a glasshouse by sowing 1 Ro seed per unit in a modular seed tray containing a peat based compost. The seed trays were watered daily for a period of 2-3 weeks prior to being hardened off outdoors. Seedlings were transplanted by hand (17 May 1989) into a field which had previously received 30 kg per hectare N, 30 kg per hectare $P_2 O_5$ and 30 kg per hectare of K_2O. The field was divided into three blocks surrounded by guards rows of Norlin. The 49 lines chosen in this study were randomly arranged in plots in a lattice square design. In each block, control lines of Norlin were similarly sown as transplants to a ratio of 5 lines to one control. Each plot consisted of 50 plants occupying two adjacent rows 1.5 m in length with an inter row gap of 10 cm and adjacent rows were separated by a 50 cm discard.

2.4.2. Field trial 1990

20 somaclonal lines R1's derived from Norlin evaluated in 1989 were direct sown (24 April 1990), in the same field as 1989 and with the same fertiliser addition (24 April 1990) to produce the R2 generation. These lines were selected upon the basis that for one or more than one characteristics they have been significantly different from the Norlin control and the R1 generation. For each line (and Norlin control), seeds were sown directly in the field by hand in a row 3 m x 10 cm wide at a density of 800-900 plants per m^2 There were 5 blocks. Within each block the control was sown in one row for every 4 test lines rows.

2.4.3 Assessments and statistical analysis

Throughout the 1989 growing season assessments were carried out on the R1's and the Norlin control. For growing plants a random sample of 10 plants from 50 was taken as being representative of the character. The following assessments were carried out:

Tiller number, flowering date (days after planting), stem height in flower, end of flowering date (days after planting), stem height at end of flowering, maturity stages, final plant height, seed yield per 10 plants, 1000 seed weight and oil content as determined by nuclear magnetic resonance spectroscopy (NMR).

During 1990, a random sample of 10 plants from 50 was taken as representative for each character in a replicate namely, flowering date, stem height in flower, stem height at final harvest, seed yield per 100 plants and 1000 seed weight. The data were analysed statistically using a residual maximum likelihood test (Genstat 5.2 copyright Lawes Agricultural trust). The object of the test was to compare the individual somaclonal lines with each other and also with the Norlin control from which all lines were derived.

A regression analysis was conducted (Genstat 5.2) to provide estimates of heritability for R1-R2 generations. Within the 20 somaclonal lines common to both the R1-R2 generations regressions was prepared for final plant height, flowering date, seed yield and 1000 seed weight.

CHAPTER 3

RESULTS

3. RESULTS

3.1 Callus culture

After 20 or 30 days of culture, the six cultivars selected produced bright green callus tissue approximately 5 to 10 times the size of the initial explant. The growth of Norlin, a linseed cultivar, in culture was especially vigorous. Within the cultivars there emerged three distinct types of callus growth. First, Norlin produced many shoots from along the length of the hypocotyl segment in addition to the production of callus initiated from the end section of the hypocotyls. Second, the cultivars Berber (fibre flax) and Linda (linseed) could be described as embryonic types since they produced 25 to 100 shoots from the initial callus tissue. Third, Lidia, Natasja, and Saskia (fibre cultivars) produced essentially callus tissue only without regenerant tissue. The shoots of Linda and Berber were chlorotic on the callus induction medium but became green thereafter when transferred to a medium free of growth hormones.

The experiment also showed that all the cultivars could be maintained on the same medium for an extended period (up to seven weeks); however, even after three weeks all the cultivars progressively lost vigour. Consequently, it was impossible to regenerate shoots from these calli.

On the regeneration medium, Lidia, Natasja and Saskia (fibre flax cultivars) did not produce any shoots in contrast to the calli of Norlin (linseed) which produced shoots rapidly. For Norlin, some 50% of the callus pieces in culture produced shoots and the number of shoots per piece was very variable (2 to 17 shoots per callus). All the shoots were healthy and once successfully

rooted could be grown on to mature plants. Berber (fibre flax) and Linda (linseed) produced shoots directly in the callus medium and it was essential to transfer them on to the rooting medium directly from which they too ultimately produced mature plants (ca. 30% of calli regenerated).

The rooting of the shoots for all cultivars was difficult at first, if no growth hormone was added to the culture medium. Addition of IAA allowed root formation within 2 weeks after transfer. In these conditions all the genotypes could be rooted. The conditions of culture (temperature) were critical to obtain sufficient shoot elongation and subsequently rooting of the shoots. If the shoots were too small, the action of the auxin induced callus formation to take over.

In a second experiment (see 2.1.3), the capacity of flax genotypes (Saskia and Natasja) to regenerate was determined. Both genotypes responded to the media in the similar manner. The level of regeneration was low (0-6%) for all treatments (Table 3.1) except when MS + BA(1) + NAA (0.02) was used giving between 15-21% regeneration. Calli plated on medium supplemented with KI alone were light green and slow growing. A combination of KI + IBA inhibited callus growth.

By contrast, the calli plated on MS+BA were bright green in colour and grew vigorously. The behaviour of the linseed cultivar Linda was determined in a subsequent experiment. As previously described, shoot formation occurred after 30 to 40 days of callus growth. Pieces of calli were plated on regeneration medium to check their ability to produce shoots. Regeneration

Table 3.1 Regenerative ability of flax cultivars *in vitro*

Genotype	Media *	Total number of calli	Number of embryogenic calli	% Regeneration
Saskia	MS+BA(1)	90	3	3.3
Saskia	MS+KI(1)	90	1	1.1
Saskia	MS+KI(1)+IBA(2)	90	3	3.3
Saskia	MS+BA(1)+NAA(.02)	20	3	15.0
Natasja	MS+BA(1)	90	6	6.6
Natasja	MS+KI(1)	90	1	1.1
Natasja	MS+KI(1)+IBA(2)	90	0	0
Natasja	MS+BA(1)+NAA(0.2)	122	26	21.3

* concentrations in parenthesis are in mg/l

capacity was less than for the linseed types Norlin (13.3% vs 52.3 %, Table 3.2).

Observations on the morphological features of shoots grown in jars were noted (Tables 3.3-3.6). Plantlets of Norlin, Linda, Lidia and Berber showed differences in shoot pigmentation. In Linda and Norlin three types of pigmentation could be found, translucent, red and pigmented. Norlin had the highest percentage of translucent shoots (8.8 % Norlin, 3.3% Linda) whereas Linda showed a great percentage of pigmented and red shoots (pigmented 6.3%, red 2.7% vs pigmented 1.01, red 0.8 for Norlin). In addition, Norlin, Linda and Berber produced a few dwarf plants. Changes in flower and seed colour were also noted for all cultivars. Linda and Norlin produced a few sterile plants.

Observations on the morphological characteristics of selected Ro plants derived from Linda, Natasja, Norlin and Saskia were recorded for total plant height, seed number and 1000 seed weight (Table 3.7). The Ro plants for all the genotypes were very variable in height and, seed number and to a lesser extent for 1000 seed weight. Differences betwen batches and origin were found for the above characteristics. For example, for the cultivar Linda batch 2 plants obtained from callus regeneration produced heavier seeds and taller plants than those derived from adventitious shoots. Plants from batches of Norlin derived callus were quite dissimilar for seed number, seed weight. The 1000 seed weight was fairly consistent for each cultivar irrespective of the original type of callus. Linseed genotypes Linda and Norlin had the largest seeds.

Table 3.2 Regenerative ability of linseed cultivars *in vitro*

Genotypes	Origins *	Total calli number	Embryonic calli number	% Regeneration
Linda	1	165	22	13.3
Linda	2	197	51	25.9
Norlin	1	175	91	52.3
Norlin	2	220	108	49.1

* origins: 1 = callus tissue, 2 = adventitious shoots from explant

Media: growth = MS + BA(1) + NAA (0.02)

regeneration = MS + BA (1)

Table 3.3 Summary of the morphological features noted in regenerant plants* (Ro) from explants of the linseed cultivar Norlin.

Morphological feature	Phase of occurrence	% Occurence
Leaf Characteristics		
Colour: light green-blue green	Seedling (4 wks)	
Shape: long narrow-oblong	Seedling - maturity	
Colour: vitreous, red, yellow, spotted, mottled	Seedling (4-8 wks)	9/1/1 1/0.3
Height: dwarf	Maturity	2
Axillary buds:	Maturity	44
Flower characteristics		
Style colour: white, purple blue/white, pink	Maturity	69/4 15/11
Filament colour: white	Maturity	100
Sterility	Maturity	7
Seed colour		
light & dark brown black, yellow variegated	Maturity	91/9 17/17 17

* Observations from an initial population of 365 seedlings to 27 mature plants

Table 3.4 Summary of the morphological features noted in regenerant plants* (Ro) from explants of the linseed cultivar Linda

Morphological feature	Phase of occurrence	% Occurrence
Leaf characteristics		
Colour: light green-blue green	Seedling (4 wks)	
Shape: long narrow-oblong	Seedling - maturity	
Shoot characteristics		
Colour: vitreous, red red spotted, yellow	Seedling (4 - 8 wks)	3/3 6/1
Height: dwarf	Maturity	1
Flower characteristics		
Style colour: white, light purple blue/white, pink, dark purple	Maturity	8/8 8/8 66
Filament colour: white blue	Maturity	25 75
Sterility	Maturity	8
Seed colour		
brown & yellow	Maturity	92/8

* Observations from an initial population of 335 seedlings to 12 mature plants

Table 3.5 Summary of the morphological features noted in regenerant plants* (Ro) from explants of the fibre flax cultivar Berber

Morphological	Phase of occurrence	% Occurrence
Leaf characteristics		
Colour: light green-blue green	Seedling (4 wks)	
Shape: long narrow-oblong	Seedling - maturity	
Shoot characteristics		
Colour: vitreous, red, red spotted	Seedling (4 - 8 wks)	27/5 10
Height: dwarf	Maturity	10
Flower characteristics		
Style colour: white	Maturity	100

* Observations from an initial population of 111 seedlings to 2 mature plants

Table 3.6 Summary of the morphological features noted in regenerant plants * (Ro) from explants of fibre flax cultivar Lidia

Morphological feature	Phase of occurrence	% Occurrence
Leaf characteristics		
Colour: light green-blue green	Seeding (4 wks)	
Shape: long narrow-oblong	Seedling - maturity	
Shoot characteristics		
Colour: vitreous, red spotted	Seeding (4 - 8 wks)	19/11
Flower characteristics		
Style colour: Pink	Maturity	100
Seed characteristics		
Seed colour: light brown	Maturity	100

* observations from an initial population of 27 seedlings to 1 mature plant

Table 3.7 Summary of selected plant characteristics in Ro plants derived from linseed and fibre flax cultivars

Genotypes	Plant number	Batch number	Height (cm)		Seeds/ plant		1000 Seed weight (g)	
Linseed genotypes:								
Linda[a]	15	1	58.3	(18.9)	41.1	(32.5)	5.2	(0.8)
Linda[a]	35	2	53.3	(17.7)	15.0	(17.9)	5.1	(1.2)
Linda[c]	25	2	63.6	(11.3)	27.5	(17.4)	6.4	(1.2)
Norlin[c]	30	1	90.6	(13.7)	220.8	(135.2)	3.3	(1.1)
Norlin[c]	38	2	96.5	(13.1)	148.6	(87.0)	4.2	(1.2)
Norlin[a]	35	2	87.7	(17.4)	129.6	(68.4)	4.7	(0.6)
Fibre genotypes:								
Natasja[a]	21	2	102.2	(18.3)	112.0	(134.0)	3.6	(0.6)
Saskia[c]	13	2	99.6	(8.3)	111.8	(33.0)	3.4	(0.5)

* Figures in parenthesis represent standard error of the mean

** Explant source: $_c$ = callus tissue, $_a$ = adventitious shoots from explant

Norlin Ro plants showed mutation for seed coat colour (fawn, brown, black). The Chi-square test was only carried out on segregating lines for fawn, brown and black (Table 3.8) and showed that in two out of ten genotypes seed coat colour was inherited in a simple Mendelian manner (3:1 or 1:2:1).

3.2 Suspension culture

Only cell suspensions of Saskia could be established rapidly, within a week of inoculation of the media. Not all the media were suitable for cell growth. For example, on media supplemented with 1 mg/l of BA +0.50 mg /l of NAA cell lines died out after one week and were subsequently discarded from the experiment. Differences in growth were noted on the remaining media. Cells maintained on MS+1 mg/l of BA +0.02 mg/l of NAA grew twice as fast as those maintained on MS+1 mg/l of BA.

The calli from both media were morphologically different 3 weeks after plating. Calli grown and regenerated on MS+BA (1) were variable in colour (bright green or yellow) and they were bright green if plated on MS+1 mg/l of BA +0.50 mg/l of NAA. Those grown on MS+1 mg/l of BA + 0.02 mg/l of NAA presented the same morphological variation. Only calli initiated on MS+1 (BA) and plated on the same media, regenerated shoots after 3 weeks (1-6%). None of the calli obtained from MS+BA (1)+NAA (0.02) and plated on either medium produced shoots. Also, shoot regeneration capacity decreased with the time in culture. After 21 days, the calli had lost their ability to regenerate (Table 3.9).

Table 3.8 Analysis of seed colour segregation in Ro lines of Norlin-derived linseed genotypes

	Origin *	Seed colours	Expected ratio	X^2 Analysis ** X^2	CDF	P
88Nor228	1	90 black 25 brown	1:3	0.21739	0.3590	ns
88Nor281	1	51 fawn 52 black	1:1	0.00970	0.0785	ns
88Nor 285	1	27 fawn 70 brown	1:3	0.97087	0.6754	ns
88Nor 287	1	19 fawn 176 brown	1:3	0.21739	0.3590	ns
89Nor19	1	24 fawn 114 brown	1:3	4.2609	0.9610	ns
89Nor10	2	53 fawn 69 brown 33 black	1:2:1	7.0258	0.9920	sig
89Nor11	2	16 fawn 63 brown	1:3	0.94937	0.6701	ns
89Nor21	2	31 fawn 77 brown	1:3	7.333	0.9932	sig
89Nor31	2	31 fawn 45 brown	1:3	2.5789	0.8917	ns

* Explant source: 1 = callus tissue, 2 = adventitious shoots from explant

** X^2 = Chi-square value, CDF = Cumulate density function

P = probability ns = not significant (P=0.05) sig = significant (P = 0.05)

Table 3.9 Regenerative ability of microcalli derived from the fibre flax cultivar Saskia

Media:	Days in culture	Number of Calli on Regeneration media *		Number of Regenerants/ Regeneration level **	
		1	2	1	2
MS+BA(1)	7	67	72	4 (6.0)	0
	14	67	65	1 (1.5)	0
	21	57	39	0 (0)	0
MS+BA(1) +NAA(0.02)	7	138	142	0	0
	14	140	104	0	0
	21	106	105	0	0

* Regeneration media used: 1 = MS+ 1 mg/l of BA, 2 = MS+ 1 mg/l of BA + 0.50 mg/l of NAA

** Regeneration level: figures in parenthesis indicate percentage of regeneration

3.3 *In vitro* selection

Identification conducted by CMI confirmed that all strains isolated were *Fusarium oxysporum,* but forma specialis could not be determined morphologically. Instead, a seedling infectivity study was carried out.

All the seeds germinated close to 100% and disease progressed rapidly with greater levels of infection caused by F60 strain (Table 3.10). There was no significant difference between the percentage of healthy plants present at 20 and 40 days after planting. There was a significant difference in disease development (P=0.01) between the two runs of the experiment where the aggressive strain was used.

Under the less intense disease pressure of the A10 strain, none of the somaclonal lines showed improved resistance to *Fusarium* but lines 12 and 14 showed lower level of resistance compared to the parent Norlin in run 1. Only line 15 showed improved resistance to the F60 strain in run 1 but this performance was not repeated in the second run. Under the intense selection presure from the F 60 strain in run 1, lines 5, 8 and 14 showed complete susceptibility.

3.4 Field trials (Norlin R1)

Within the R1 generation it was apparent that lines differed statistically (P=0.05) from the control (Norlin) only in final plant height, flowering date,

Table 3.10 Responses of some cultivars/somaclonal lines of linseed to two strains of *F. oxysporum* f.sp. *lini*

Somaclone/ cultivar identity	Mean percentage of healthy plants (angular transformation)			
	Run 1 Strain F60	Run 2 Strain F60	Run 1 Strain A10	Run 2 Strain A10
1	8.3	62.4	76.7	55.3
2	9.7	42.1	90.0	53.1
3	9.2	49.9	67.5	53.0
4	13.8	46.7	79.9	51.7
5	0	41.4	90.0	65.8
6	14.1	30.6	76.2	90.0
7	4.6	55.5	80.8	59.6
8	0	37.4	79.9	76.6
9	32.1	54.6	76.7	71.6
10	21.0	34.2	75.1	73.4
11	10.4	41.9	65.3	78.8
12	25.7	42.1	40.9	61.4
13	37.0	51.1	73.4	70.7
14	0	43.3	27.1	81.7
15	45.0	35.4	55.2	90.0
Norlin	18.8	48.5	76.0	63.3
Norlin R_0	22.5	36.5	90.0	90.0
Regina (susceptible)	0	0	23.5	16.0
L.S.D. (P = 0.05)	23.5	20.2	28.4	20.0

seed yield, oil yield and 1000 seed weight (Figure 3.1). With the exception of 1000 seed weight, the value recorded for each character was close to the mean of the 49 somaclonal lines. By contrast, the 1000 seed weight of 70% of the somaclonal lines were significantly lower than the control (P=0.05).

Three lines (Nor 243, Nor 23 and 43) were significantly shorter (P=0.05) than the control (by 4-5 cm) while lines Nor 228, 186 and 54 were taller (by 2-3 cm). Nor 228, 184, 47, flowered 2-3 days later than the control (P=0.05). Seed yield was lower (P=0.05) in Nor 257 (by 7-8g:10 plants). Final oil yield (oil % x seed yield) was significantly lower (P=0.05) in Nor 229 TB and 257 (4-4.5g/10 plants). Twelve lines showed 1000 seed weight significantly lower than the control.

3.5 Field trials (Norlin R2)

Final plant height, flowering date, seed yield and 1000 seed weight for the 20 somaclonal lines and the control are presented in Figure 3.2. Four lines (Nor 44,15, 45 and 184) were 2-6 cm shorter than the control while Nor 5 and 278 were taller by 2-4 cm (P=0.01). Flowering was significantly earlier (P=0.01). In an extreme case (Nor 6) flowering commenced 6 days earlier than the control. The distribution of the seed yield data showed that with one exception (Nor 182) the somaclonal lines were lower in yield than the control (P=0.01). Only two lines (Nor 182 and 45) had heavier 1000 seed weight than the control while 50% of the test lines were lighter (P=0.05).

Figure 3.1 Distribution of selected characteristics (total plant height, flowering date, seed yield, oil yield and 1000 seed weight) in 47 R1 somaclonal lines derived from Norlin (control). The performance of the control within a category is marked by shading. Lines showing a characteristic which is statistically different from the control ($P>0.05$) are marked with an asterisk.

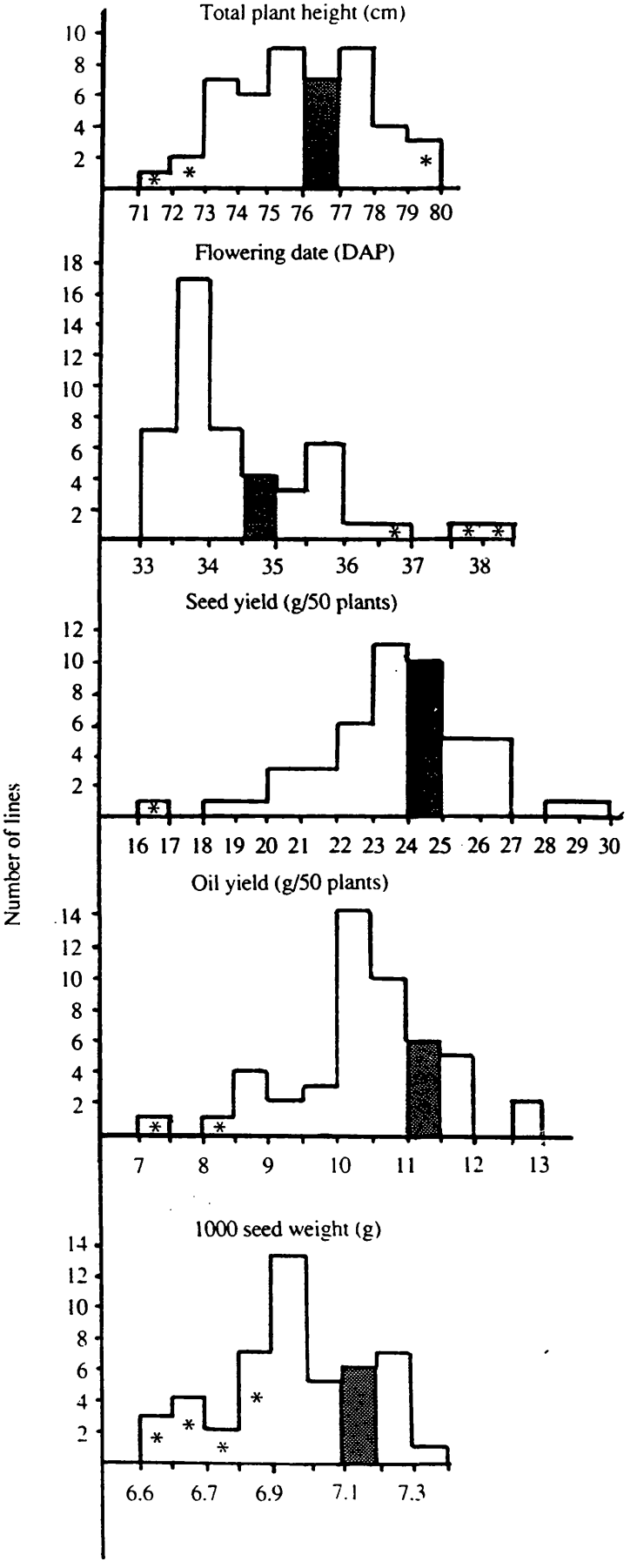
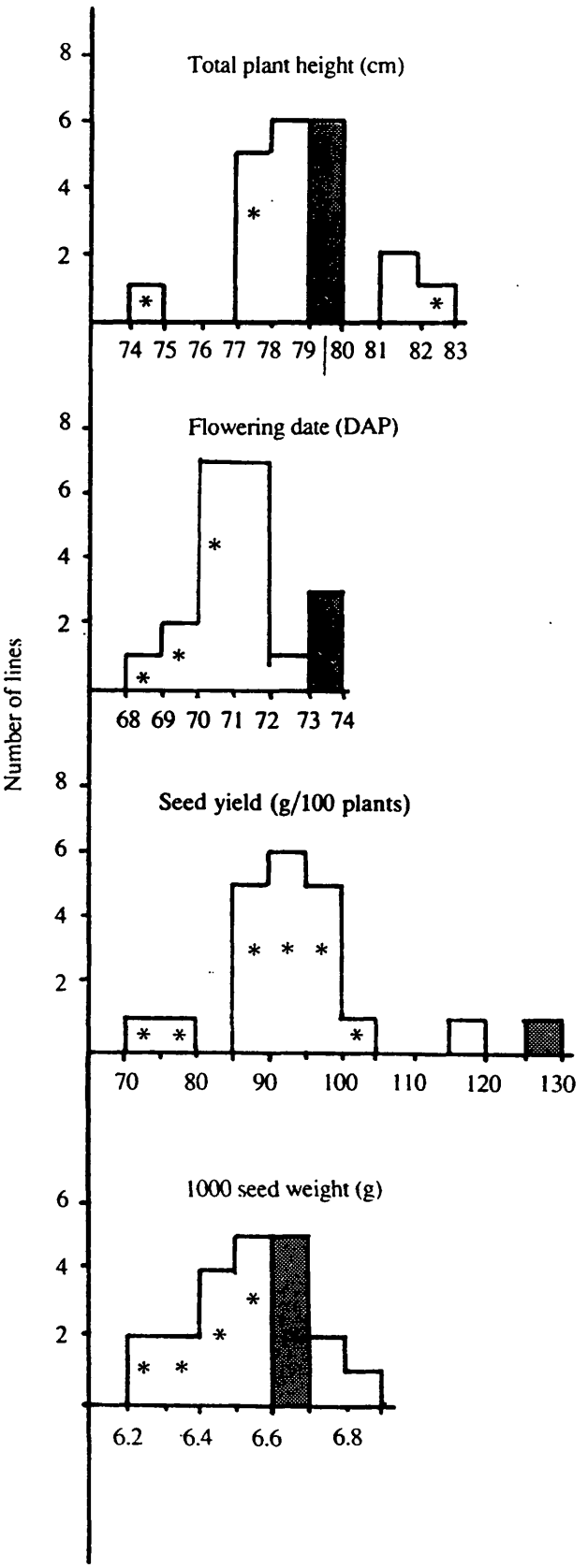

Total plant height (cm)
10
8
6
4
2
71 72 73 74 75 76 77 78 79 80
Flowering date (DAP)
18
16
14
12
10
8
6
4
2
33 34 35 36 37 38
Seed yield (g/50 plants)
12
10
8
6
4
2
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Oil yield (g/50 plants)
14
12
10
8
6
4
2
7 8 9 10 11 12 13
1000 seed weight (g)
14
12
10
8
6
4
2
6.6 6.7 6.9 7.1 7.3
Number of lines

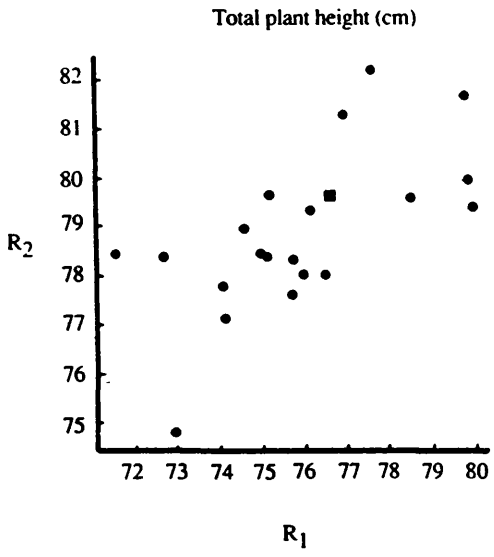
Figure 3.2 Distribution of selected characteristics (total plant height, flowering date, seed yield and 1000 seed weight) in 20 R2 somaclonal lines derived from Norlin (control). The performance of the control within a category is marked by shading. Lines showing a characteristic which is statistically different from the control ($P>0.05$) are marked with an asterisk.

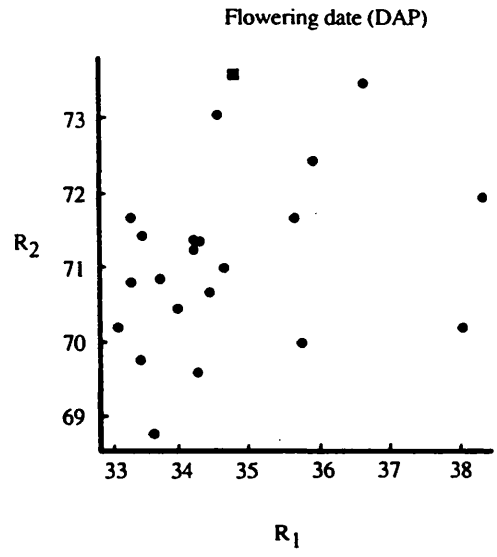

Total plant height (cm)
Flowering date (DAP)
Seed yield (g/100 plants)
1000 seed weight (g)
Number of lines
8
6
4
2
74 75 76 77 78 79 80 81 82 83
68 69 70 71 72 73 74
70 80 90 100 110 120 130
6.2 6.4 6.6 6.8

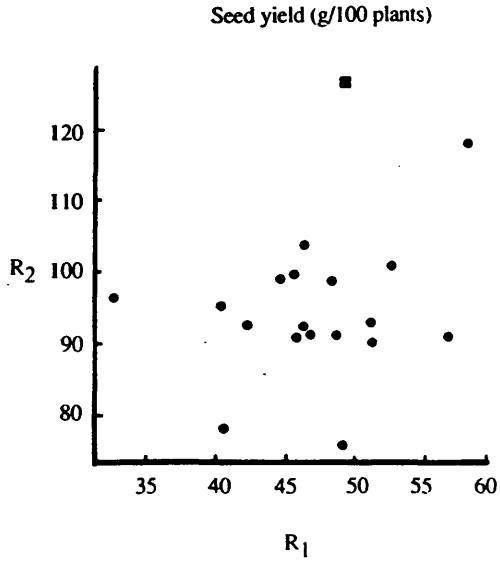
3.6 Heritability estimates

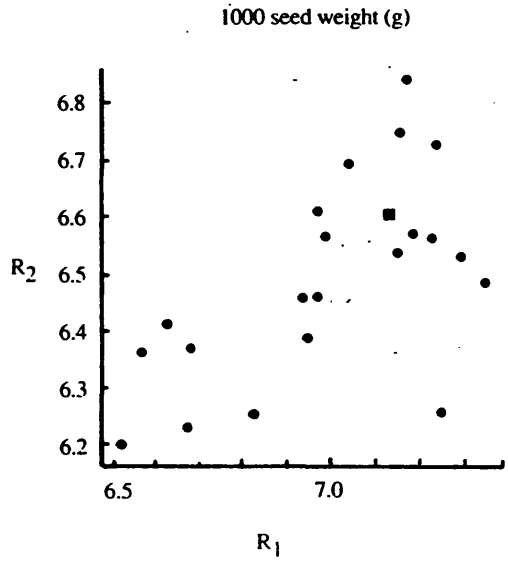
Correlation between parent offspring generations (R1/R2) indicate moderately high heritabilities for final plant height (r=0.64) and 1000 seed weight (r=0.61) but low heritability for flowering date (r=0.31) and seed yield (r=0.28). Scatter diagrams (Figure 3.3) clearly show that the broad ranking of final plant height and 1000 seed weight in relation to the control Norlin was similar in both generations.

Figure 3.3 Scatter plots showing a regression analysis of selected traits in 20 R1 and R2 somaclonal lines [●] derived from Norlin [■] (control). Correlation coefficients: total plant height (r=0.64), flowering date (r=0.31), seed yield (r=0.28), 1000 seed weight (r=0.61).


Total plant height (cm)
82
81
80
79
78
77
76
75
R_2
72 73 74 75 76 77 78 79 80
R_1


Flowering date (DAP)
73
72
71
70
69
R_2
33 34 35 36 37 38
R_1


Seed yield (g/100 plants)
120
110
100
90
80
R_2
35 40 45 50 55 60
R_1


1000 seed weight (g)
6.8
6.7
6.6
6.5
6.4
6.3
6.2
R_2
6.5 7.0
R_1

CHAPTER 4

DISCUSSION & FUTURE STUDIES

4. DISCUSSION & FUTURE STUDIES

4.1 Callus growth and regeneration

Callus was produced readily by all the cultivars using the MS based medium. The major feature where the cultivars showed differences was in terms of the type of callus produced and the regeneration potential of the callus. Norlin showed a special capacity to produce regenerant shoots on callus induction medium. The cellular origin of the regenerant shoots is likely to be the epidermal cells of the hypocotyl sections as described by Gamborg and Shyluk (1976). Embryonic callus produced by Berber and Linda was most probably derived from the cortex and cambium cells. Again these cells had the ability to regenerate shoots from the callus tissue. By contrast, neither Natasja or Saskia were induced to regenerate shoots on callus medium. It is possible that these cultivars may respond to culture on MS medium modified to include cytokinin e.g zeatin (0.25-1.0 μM, as per Gamborg and Shyluk (1976) or kinetin (1 mg/l) as per McHughen and Swartz (1984).

To different degrees the cultivars Berber, Lidia, Linda and Norlin were induced to produce shoots and roots where callus was placed on regeneration medium. The final production of flowering plants was influenced by the capacity of the plants to root in the media chosen (MS and B5) but also to be nursed from the closed jars out into the control environment. Certainly half strength B5 medium enhanced rooting to a greater degree than MS. However, these experiments have showed that

half strength B5 medium incorporating 175 µg/l provided greatly enhanced rooting with a small reduction in shoot vigour.

In addition, it was shown in these studies that regenerant plants can be saved by taking cuttings and rooting these in a proprietary hormone based rooting powder containing NAA plus captan. The cuttings then root successfully in the normal peat/perlite compost while maintaining the shoots under humid conditions with plastic domes. Previous reports by Dorrell (1974) confirm the ability of *L. usitatissimum* to be propagated by stem cuttings.

The efficiency of regeneration within each cultivar was related to the genotype. In addition, there seemed to be a real physiological difference between the oil and fibre types with regard to their reaction to the selection of culture media which have been tested in these experiments. Such a comparison has not previously been documented. Clearly if more time was devoted to optimising the composition of the growth and regeneration media it may be possible to improve the regeneration efficiency of fibre bearing cultivars Lidia, Natasja and Saskia.

It was apparent that the Ro material was very variable in a range of morphological characters including leaf shape, seed and flower colour, stem habit and sterility. These characteristics were not unexpected and indeed are similar to those previously reported for somoclonal variation in general (Vasil, 1988). Several of the characteristics noted were even transient within that one generation and as will be discussed later (4.4) did not show in subsequent generations (R1 and R2).

One important feature of this work reveals that it is possible to produce sufficient quantities of Ro seed from *in vitro* systems (particularly linseed) to enable successive generations of field trialling to assess the practical value of somaclonal variation. Clearly in the time available for this project is was not possible to take every somaclonal line from every cultivar forward for field trial, instead the most prolific cultivar, Norlin was chosen (see section 4.3).

4.2 Suspension culture

There was a clear difference in the efficiency of the suspension culture system compared to that discussed in the previous section (4.1). While regeneration efficiencies from callus cultures were in the realms of 15-50%, by comparison regeneration from suspension cultures was at best 6.0%. In order to pursue the success of the callus culture work and to ensure its evolution through to the field trials, it was decided to limit the effort devoted to the suspension culture studies.

It is obvious that for *in vitro* selection studies (herbicide or disease toxins), further work would need to be carried out to improve the efficiency of regeneration achieved after 2-3 weeks of culturing. Where regeneration is not required in the studies e.g biochemical assay or characterisation these fibre bearing cultivars which we have tested (e.g. Saskia) can be used to produce fine vigorous suspension cultures. By contrast, it was especially difficult to produce good fine suspensions from linseed cultivars. Again this emphasises the physiological differences of the oil and fibre types *in vitro.*

4.3 *in vitro* selection

In vitro selection for improved disease resistance has been achieved in some crops (see review by Daub, 1986); however, in the linseed cultivar Norlin, no such improvements were apparent (3.3). The selection protocol is difficult to standardise in terms of defining the selection pressure. In other words, inoculum is cultured, filter sterilised and diluted in a standard way but without making a quantitative determination of the components of the crude extract with toxins. Further characterisation of the toxin components is required if we are to understand the action of the *in vitro* selection process.

The selection process achieved limited success in terms of identifying new or improved forms of *Fusarium* resistance. Under the disease pressure of the aggressive F60 strain only one line (15) appeared to have comparable or improved resistance compared to the Norlin parent (Table 3.10). Where the less aggressive strain A10 was used for *in vitro* selection, there was much less evidence to suggest that disease resistance was in fact breaking down. The majority of the lines showed comparable level of resistance to the parent Norlin.

Somaclonal lines are difficult to screen accurately since the genetic material is likely to be variable in the Ro generation. These somaclonal lines did not show consistency of improvement in disease resistance. The test described was not reproducible due to fluctuations in the temperature under glasshouse conditions which influence the rate and severity of disease

development. Still, the method could be usefully applied and reproducibility improved in a controlled environment facility.

The only other studies to report *in vitro* selection in linseed are those from Jordan and McHughen (1987) for herbicide resistance and McHughen and Swartz (1984) and McHughen (1987) for salt tolerance. Field assessments of the putative salt-tolerant line ultimately showed that the agronomic performance of the somaclonal variety was not salt-tolerant *per se* but showed an earlier pattern of growth as compared to the parental variety (Rowland, McHughen and McOnie, 1988).

4.4 Field trials

In this work early attempts to assess the morphological characters in the Ro generation showed that Norlin displayed variation in leaf, shoot, flower and seed characteristics (Table 3.3). The value of such observations can be limited since it is well known that such variation is frequently short-lived and of little practical value (Vasil, 1990). Furthermore, it is not possible to make a fair comparison of individual plants as they were raised asynchronously in the controlled environment over a period of 12 months. Such assessments are problematic as a consequence of transient morphological characteristics and in the difficulty in separating genotypic and environmental effects on the phenotype of Ro somaclones (Engler and Grogan, 1984).

Early predictions suggested that tissue culture would provide an unexpectedly rich and novel source of genetic variability (Larkin and Scowcroft, 1981). In practice however little of the variation found in cell

cultures is recovered in regenerated plants. Almost all of it is either useless or similar to the variation recovered after sexual crossing or mutagenesis (Vasil,1990).

In this study, limited variation was recovered from the R1 and the R2 generations (3.4, 3.5). Many of the somaclonal lines showed significant differences from the Norlin control in only one or two characters. Evans (1989) suggested that the value of somaclonal variation may indeed be where a new useful characteristic appears in an otherwise unchanged genotype. Within the traits which showed somaclonal variation, the reduction of stem height (Nor 44) and earliness of flowering (various lines) could be of value in a breeding programme. The trait which seemed to be most detrimental in the new somaclonal lines was the low 1000 seed weight (Nor 186, 228, 229TA, 229TB, 254, 257). Mean individual seed weight is considered to be a principal determinant of the final seed yield and a trait which is particularly stable (Obeid, Machin and Harper, 1967). As a consequence of generally low 1000 seed weight none of the somaclonal lines showed any improvement in yield over the Norlin control .

It was not surprising to find that characteristics did not remain stable compared to the control between the R1 and R2 generations (3.6). In these early generations, somaclonal variation is known to demonstrate epigenetic non-heritable changes which can only be assessed with accuracy after several sexual generations (Evans and Bravo, 1986). In addition it should be noted that this assessment of somaclonal variation in linseed was made at one location with only one parental genotype over two rather similar seasons. Obviously there would be merit in continuing these assessments

at more than one location since traits which may be important in another region e.g. resistance to *Fusarium oxysporum* f.sp. *lini* might become apparent under the appropriate selection pressure. Additional studies carried out at SAC, Auchincruive (Marshall, 1993, Pers. comm.) have shown that the somaclonal variation expressed is influenced for both oil and fibre bearing flax by the parent genotype chosen (unpublished results). It is evident that somaclonal variation experiments of this type especially in the early generations are unlikely to hasten the conventional breeding and selection process in flax.

The inheritance of the trait will also influence the likely success of identifying lines which possess the desired traits. Thus variation in traits inherited in a simple fashion e.g. by simple genes which are highly heritable (resistance to flax rust and mildew) are likely to be relatively easy to select. By contrast, polygenically-inherited traits such as flowering date, plant height, yield, 1000 seed weight are much more difficult to select from in early generations (R2) because the lines may not yet be homozygous so that genes are not fully expressed. In addition, it is confirmed in this study that the heritability of these traits is quite variable (between 28 % and 64%) but of similar order to those described by Fouilloux (1989) for classical breeding programmes. Only further years of evaluation will resolve these questions of selection for polygenically inherited traits.

4.4 Assessing somaclonal lines - practical considerations

In order to characterize the variation which exists in the somaclonal lines it is essential to have a properly organised statistical layout. In the early

generations (Ro and R1), there is invariably a shortage of seed especially in a species like flax so that proper replications cannot be conducted. This restriction makes it all the more difficult to assess variation in the R1's with any reliable degree of certainty. In these experiments the risk of direct sowing of the Ro in soil was avoided to prevent potentially serious seedling mortalities due to disease. As events turned out the spring weather was so favourable that Ro's could have been safely direct sown. The burden of seed borne disease should not be underestimated since our R1's were heavily contaminated with *Alternaria linicola* and *Phoma exigua* var. *linicola.* The provision of an adequate number of control lines is also an important feature in the assessment procedure. During the first field trials (1989) the results analysed suggested that improved precision could be obtained by increasing the frequency of control cultivars. In 1990, the ratio of somaclonal lines to control was raised to 4:1. Recent publications suggest a general rule that the square root of the number of test lines should be planted with controls.

4.5 Future studies

The research conducted in this programme has produced a valuable range of somaclonal lines which can be used in future studies. It is clear that none of the somaclonal lines or lines selected via *in vitro* protocols would be immediately of value to the plant breeder. Hovever, there are other scientific opportunities to use these somaclonal lines in genetical molecular and pathological studies. In particular, these lines provide partly characterised mutants e.g. changes from disease resistant to disease suceptible and changes in polygenically inherited characteristics such as

seed weight and yield. These lines may occasionally be similar to near isogenic lines, e.g. genetically similar to the original parent cultivar but with changes in one or two traits. This type of material will be especially useful in future programmes to characterise the genome of flax. Restriction fragment length polymorphism (RFLP) and other molecular mapping studies e.g. Amplified fragment length polymorphism (AFLP) could compare somaclonal lines and parents to locate and characterise traits of economic importance.

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